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| (54) Title: TGF- β 1/ β 2: A NOVEL CHIMERIC TRANSFORMING GROWTH FACTOR-BETA (57) Abstract The present invention relates to the production of large quantities of a novel chimeric TGF- β , termed TGF- β 1/ β 2, by eu- caryotic host cells transfected with recombinant DNA vectors containing the TGF- β 1/ β 2 precursor coding sequence controlled by expression regulatory elements. Simian TGF- β 1 cDNA (Sharples et al., 1987, DNA 6:239-244) was modified so that the nucleo- tides encoding amino acid residue numbers 9-13, 17, 19, 25 and 26 of the mature TGF- β 1 sequence were hanged to the nucleo- tides encoding the corresponding amino acids of the mature TGF- β 2 structure. Simian codon usage was maintained. The chimer- ic TGF- β 1/ β 2 of the invention induces effects on the proliferation of vascular endothelial cells equivalent to those induced by TGF- β 1 (see Section 7., <i>et seq.</i>). | | |

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TGF- β 1/ β 2: A NOVEL CHIMERIC TRANSFORMING GROWTH FACTOR-BETA1. INTRODUCTION

The present invention relates to a novel chimeric transforming growth factor-beta termed TGF- β 1/ β 2 (or TGF- β 5), to nucleotide sequences and expression vectors encoding TGF- β 1/ β 2, and to methods for the production of TGF- β 1/ β 2. The invention is exemplified by the production and secretion of TGF- β 1/ β 2 by CHO cells transfected with expression vectors encoding a chimeric TGF- β 1/ β 2 precursor gene. The chimeric gene product possesses TGF- β biological activity.

2. BACKGROUND OF THE INVENTION

Transforming growth factor-Beta (TGF- β) is a member of a recently described family of polypeptides that regulate cellular differentiation and proliferation. Other members of this family include Mullerian inhibitory substance (Cate et al., 1986, Cell 45:685-698), the inhibins (Mason et al., 1985, Nature 318:659-663) and a protein predicted from a transcript of the decapentaplegic gene complex of *Drosophila* (Padgett et al., 1987, Nature 325:81-84).

Four types of TGF- β have been identified and designated TGF- β 1, TGF- β 2, TGF- β 1.2, and TGF- β 3. The first described type, TGF- β 1, consists of two identical disulfide linked subunits having molecular weights of 13,000 (Assoian et al., 1983, J. Biol. Chem. 258:7155-7160; Frolik et al., 1983, Proc. Natl. Acad. Sci. USA 80:3676-3680; Frolik et al., 1984, J. Biol. Chem. 260:10995-11000). It has been purified from several tissue sources including placenta (Frolik et al., 1983, Nature 325:81-84), blood platelets (Childs et al., 1982, Proc. Natl. Acad. Sci. USA 79:5312-5316; Assoian et al., 1983, J. Biol. Chem. 258:7155-7160) kidney (Roberts et al., 1983, Biochemistry 22:5692-5698), and demineralized bone (Seyedin et al., 1985, Proc. Natl. Acad. Sci. USA 82:119-123). cDNA clones coding for human (Derynck et al., 1985, Nature 316:701-705), mouse (Derynck et al., 1986, J. Biol. Chem. 261:4377-4379) and simian (Sharples et al., 1987, DNA

6:239-244) TGF- β 1 have been isolated. DNA sequence analysis of these clones indicates that TGF- β 1 is synthesized as a large precursor polypeptide, the carboxy terminus of which is cleaved to yield the mature TGF- β monomer. Strong sequence homology has been found throughout the TGF- β 1 precursor protein from all of the above sources.

In the presence of 10% serum and epidermal growth factor, TGF- β 1 promotes the anchorage independent growth of normal rat kidney fibroblasts (Roberts et al., 1981, Proc. Natl. Acad. Sci. USA 78:5339-5343; Roberts et al., 1982, Nature 295:417-419; Twardzik et al., 1985, J. Cell. Biochem. 28:289-297); in the presence of 10% serum alone, it is able to induce colony formation of AKR-2B fibroblasts (Tucker et al., 1983, Cancer Res. 43:1518-1586). TGF- β 1 has also been shown to cause fetal rat muscle mesenchymal cells to differentiate and produce cartilage specific macromolecules (Seyedin et al., 1986, J. Biol. Chem. 261:5693-5695).

In contrast to its effect on cell proliferation, TGF- β 1 purified from human platelets has been shown to inhibit the growth of certain cells in culture (Tucker et al., 1984, Science 226:705-707). TGF- β 1 has also been shown to inhibit the growth of several human cancer cell lines (Roberts et al., 1985, Proc. Natl. Acad. Sci. USA 82:119-123). This inhibitory/stimulatory effect of TGF- β 1 may depend on several factors including cell type and the physiological state of the cells (for review see Sporn et al., 1986, Science 233:532-534).

TGF- β 2, like TGF- β 1, is a polypeptide of molecular weight 26,000 composed of two identical 13,000-dalton subunits which are disulfide linked (Chieffetz et al., 1987, Cell 48:409-415; Ikeda et al., 1987, Biochemistry 26:2406-2410) and has been isolated from bovine demineralized bone (Seydin et al., 1987, J. Biol. Chem. 262:1946-1949), porcine platelets (Chieffetz et al., 1987, 48:409-415), a human prostatic adenocarcinoma cell line, PC-3 (Ikeda et al., 1987,

Biochemistry 26:2406-2410), and a human glioblastoma cell line (Wrann et al., 1987, EMBO 6:1633-1636). cDNA clones coding for human and simian TGF- β 2 have been isolated (Madisen et al., 1988, DNA 7:1-8; Webb et al., 1988, DNA 7:493-497). The mature TGF- β 2 monomer is cleaved from one of two larger precursor polypeptides, the mRNAs of which may arise via differential splicing (Webb et al., 1988, DNA 7:493-497).

TGF- β 1 and TGF- β 2 share 71% amino acid sequence identity in their mature regions, and 41% identity in their precursor structures. TGF- β 3, the amino acid sequence of which has very recently been deduced from cDNA clones, appears to contain a C-terminal 112 amino acid sequence with about 80% homology to the mature monomers of TGF- β 1 and TGF- β 2 (Dijke et al., 1988, Proc. Natl. Acad. Sci. USA 85:4715-4719). TGF- β 1.2 is a heterodimeric form comprising a β 1 and β 2 subunit linked by disulfide bonds (Cheifetz et al., 1987, Cell 48:409-415).

2.1. INTRACELLULAR PROCESSING OF TGF- β 1

The amino portion of the precursor region of TGF- β 1 from human, rodent and simian sources show a high degree of homology (Derynck et al., 1985, Nature 316:701-705; Derynck et al., 1986, J. Biol. Chem. 261:4377-4379; Sharples et al., 1987, DNA 6:239-244), suggesting an important biological function may be associated with this part of the molecule. Recent studies demonstrating that this portion of the TGF- β 1 precursor is glycosylated and phosphorylated support this contention since one might assume that a cell would not go through the expense of performing these secondary modifications were it not for a specific function (Brunner et al., 1988, Mol. Cell. Biol. 8:2229-2232). These modifications may be important for dimerization of the precursor or for directing its movement out of the cell. There is evidence which suggests that glycosylation of the

precursor is involved in the transport of mature TGF- β 1 out of the cell (Purchio et al., 1988, J. Biol. Chem. 263:14211-14215).

The existence of what may either be intermediate precursor complexes involved in processing or expression artifacts in CHO cells expressing the simian TGF- β 1 gene has been reported (Gentry et al., 1988, Mol. Cell. Biol. 8:4162-4168 press; Gentry et al., 1987, Mol. Cell. Biol. 7:3418-3427). These studies revealed that the TGF- β 1 precursor synthesized by transfected CHO cells consists of pro-TGF- β 1, mature TGF- β 1, and the pro region of the precursor interlinked by disulfide bonds. Such disulfide-linked precursor complexes have also been observed in isolated latent forms of TGF- β 1 (Miyazano et al., 1988, J. Cell. Biochem. Suppl. 12(A):200; Wakefield et al., 1987, J. Biol. Chem. Suppl. 11(A):46).

Gentry et al. (Gentry et al., 1988, Mol. Cell. Biol., 8:4162-4168) have proposed the following scheme for the processing of pre-pro-TGF- β 1 in transfected CHO cells. (The amino acid position numbers referred to are from the published sequence of simian TGF- β 1 (Sharples et al., 1987, DNA 6:239-244)). According to this proposed scheme, the first step involves signal peptide cleavage at the Gly-29/Leu-30 peptide bond. This cleavage event most likely occurs co-translationally during transit of the precursor through the rough endoplasmic reticulum membrane (Blobel and Dobberstein, 1975, J. Cell. Biol. 67:835-851; Walter et al., 1984, Cell 38:5-8). Following cleavage of the signal peptide, core glycosylation units (Rothman et al., 1978, Cell 15:1447-1454) are added to pro-TGF- β 1 at each of three predicted N-glycosylation sites located at Asn-82, Asn-136 and Asn-176. The core glycosylated pro-TGF- β 1 is then sequentially processed during transit through the Golgi to yield a phosphorylated glycoprotein containing complex,

sialated oligosaccharides. At some stage during synthesis or transit, proteolytic cleavage at the dibasic residue and disulfide isomerization occurs, releasing mature TGF- β 1.

In another recent study, mannose-6-phosphate was identified in the TGF- β 1 precursor. Mannose-6-phosphate, a phosphorylated sugar analog, appears to play a fundamental role in the targeted transport and intercellular exchange of lysosomal enzymes (von Figura, 1986, Ann. Rev. Biochem. 55: 167-193). Specific receptors which recognize the mannose-6-phosphate residues of lysosomal enzymes have been identified and are essential components of the transport system. Secreted lysosomal proteins containing mannose-6-phosphate have been identified in the conditioned medium of tissue culture cells (Gal and Gottesman, 1986, J. Biol. Chem. 261:1760-1765; Capony et al., 1981, J. Cell. Biol. 104:253-262; Baumbach et al., 1984, Proc. Natl. Acad. Sci. USA 81:2985-2989; Sahagian and Gottesman, 1982, J. Biol. Chem. 257:11145-11150). It is possible that the mannose-6-phosphate residues of the TGF- β 1 precursor may direct pro-TGF- β 1 to lysosomes for proteolytic processing to yield mature TGF- β 1. Alternatively, the mannose-6-phosphate residues may function to target the cleaved TGF- β 1 precursor to lysosomes for degradation.

3. SUMMARY OF THE INVENTION

The present invention relates to the production of large quantities of a novel chimeric TGF- β , termed TGF- β 1/ β 2, by eucaryotic host cells transfected with recombinant DNA vectors containing the TGF- β 1/ β 2 precursor coding sequence controlled by expression regulatory elements. Simian TGF- β 1 cDNA (Sharples et al., 1987, DNA 6:239-244) was modified so that the nucleotides encoding amino acid residue numbers 9-13, 17, 19, 25 and 26 of the mature TGF- β 1 sequence were

changed to the nucleotides encoding the corresponding amino acids of the mature TGF- β 2 structure. Simian codon usage was maintained.

Expression vectors encoding the chimeric TGF- β 1/ β 2 precursor under the regulatory control of Simian Virus 40 (SV 40) expression regulatory elements were constructed and used to transfect Chinese Hamster ovary (CHO) cells. CHO transfectants which synthesize and secrete high levels of mature TGF- β 1/ β 2 were obtained. TGF- β 1/ β 2 expression was amplified with methotrexate and amplified transfectants secreted as much as 1mg/L mature TGF- β 1/ β 2. Acidification of the conditioned media of the CHO transfectants resulted in maximal levels of bioactive TGF- β 1/ β 2. It is believed that the high levels of mature TGF- β 1/ β 2 secreted by the transfected CHO cells results from an unusual efficiency in the proteolytic processing of the chimeric precursor protein. Such increased processing efficiency may, in turn, result from structural characteristics affected by applicants' combination of the TGF- β 1 and TGF- β 2 amino acid sequences in the amino-terminal domain of the mature TGF- β structure.

The chimeric TGF- β 1/ β 2 of the invention induces effects on the proliferation of vascular endothelial cells equivalent to those induced by TGF- β 1 (see Section 7., et seq.).

4. DESCRIPTION OF THE FIGURES

FIG. 1. Nucleotide and deduced amino acid sequence of the TGF- β 1/ β 2 hybrid protein encoded by expression plasmid p5 β /dhfr.

FIG. 2. Bioactivity of conditioned media from 5 β 41,2.5 cells. Bioactivity was measured by the growth inhibition assay of CCL-64 mink lung epithelial cells. (A) Serum-free media conditioned by 5 β 41,2.5 cells for 24 hours

was dialyzed against 0.2 M acetic acid and assayed as described in Section 6.1.3., *infra*. (B) Standard growth inhibition curve for TGF- β 1.

FIG. 3. Immunoblot analysis of proteins secreted by 5 β 41,2.5 cells. 5 β 41,2.5 cells were grown to confluence; media was dialyzed against 0.2 M acetic acid and assayed by immunoblotting under nonreducing (lane 1) or reducing (lane 2) conditions with anti-TGF- β 1₃₆₉₋₃₈₁ as described in Section 6.1.5., *infra*.

FIG. 4. Effect of various TGF- β s on vascular endothelial cells. Two-dimensional, five-day vascular cell cultures were grown in DME plus 10% FCS (control) with additions of TGF- β 1, TGF- β 2, both at 0.5 ng/ml, or TGF-5 β at concentrations of 0.05, 0.5 or 5.0 ng/ml. (A) BAEC; (B) BASMC; (C) RFC. In all cases the TGF-5 β hybrid molecule mimicked the TGF- β 1 isoform.

FIG. 5. Effect of various TGF- β s on vascular cell migration. Migrating aortic endothelial and smooth muscle cells were treated with TGF- β 1 and TGF- β 2 at 0.5 ng/ml and TGF5 β at concentrations of 0.05, 0.5 or 5.0 ng/ml. TGF-5 β mimicked the TGF- β 1 response induced on BASMCs by increasing migratory rates, while the opposite effect was seen when using large vessel endothelial cells. TGF- β 2 elicited no effect on either cell type.

FIG. 6. Angiogenic response induced by various TGF- β s. Three-dimensional RFC cultures were viewed with Hoffman interference microscopy to analyze angiogenic responses elicited by TGF- β 1, TGF- β 2, and TGF-5 β . Four-day, 3-D small vessel endothelial cultures were cryosectioned at 8 μ m and acetone fixed. Control cultures (A) were grown in DME plus 10% FCS. Treated cultures included: (B) TGF- β 1 (0.5 ng/ml); (C) TGF- β 2 (0.5 ng/ml); and TGF-5 β at (D) 0.05 ng/ml; (E) 0.5 ng/ml and (F) 5.0 ng/ml. TGF- β 1 evokes complex, branching tubular structures, while the control cultures showed minimal tube formation. TGF-5 β at 0.05 ng/ml gave an indication of

commencing neovascularization, while 0.5 ng/ml of the hybrid molecule stimulated an angiogenic response equivalent to the TGF- β 1 isoform. The highest concentration of TGF- β 2 appears to cause a slight decrease in the amount of complex branching structures which had been shown before using TGF- β 1. TGF- β 2 required a 10-fold increase in concentration (5.0 ng/ml) in order to match the neovascularization induced by TGF- β 1 and TGF- β 2 at 0.5 ng/ml. Arrows indicate tubular formations by RFCs. Magnification = 100 X.

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to TGF- β 1/ β 2, to nucleotide sequences encoding TGF- β 1/ β 2 and the TGF- β 1/ β 2 precursor, and to the production of TGF- β 1/ β 2 by recombinant DNA methods. TGF- β 1/ β 2, a novel chimeric transforming growth factor-beta, is biologically active in the standard assay used to measure TGF- β 1 bioactivity and is immunoreactive with TGF- β 1-specific antibodies. A chimera structurally comprising a combination of TGF- β 1 and TGF- β 2 amino acid sequences, the TGF- β 1/ β 2 of the invention is likely to carry a novel portfolio of biological activities, some of which may be similar or nearly identical to those exhibited by its parent molecules while others may be unique to TGF- β 1/ β 2. With regard to those bioactivities which are similar or nearly identical to those of TGF- β 1 or TGF- β 2, this new factor may provide a more effective means of inducing corresponding biological responses and its use may therefore be a desirable alternative to TGF- β 1 and TGF- β 2 in various medical applications envisioned for the TGF- β s. Such applications include but are not limited to inducing or accelerating cell proliferation and differentiation and, inhibiting cell division. Thus TGF- β 1/ β 2 may find uses in, for example, treating cancer and promoting wound healing.

The method of the invention may be divided into the following stages solely for the purposes of description: (a) generation of the coding sequence for the TGF- β 1/ β 2 precursor; (b) construction of an expression vector which will direct the expression of the TGF- β 1/ β 2 coding sequence; (c) transfection of appropriate host cells which are capable of replicating, expressing the gene and processing the gene product to produce the mature form of TGF- β 1/ β 2 and/or TGF- β 1/ β 2 precursors; and (d) identification and purification of the TGF- β 1/ β 2 precursors and the mature, biologically active TGF- β 1/ β 2.

Once a transfectant is identified that expresses high levels of TGF- β 1/ β 2 precursors and/or mature TGF- β 1/ β 2, the practice of the method of the invention involves the expansion of that clone and isolation of the gene product expressed.

The method of the invention is demonstrated herein, by way of examples in which simian TGF- β 1 precursor cDNA (Sharples et al., 1987, DNA 6:239-244) is modified so that the nucleotides encoding amino acid residue numbers 9-13, 17, 19, 25 and 26 of the mature simian TGF- β 1 sequence are changed to the nucleotides encoding the corresponding amino acids in the mature TGF- β 2 structure, while maintaining simian codon usage. The resulting chimeric TGF- β 1/ β 2 precursor coding sequence is then used to construct expression vectors which are capable of directing the synthesis of the mature TGF- β 1/ β 2 product.

The effects of TGF- β 1/ β 2 on the proliferation, migration and angiogenesis of vascular endothelial cells have been examined in vitro (section 7., et seq.) and appear to closely parallel those induced by TGF- β 1 but not TGF- β 2.

The various aspects of the method of the invention are described in more detail in the subsections below and in the examples that follow.

5.1. GENERATION OF THE CHIMERIC TGF- β 1/ β 2 CODING SEQUENCE

The nucleotide coding sequence for the chimeric TGF- β 1/ β 2 is depicted in FIG. 1. In the practice of the method of the invention, this nucleotide sequence or its functional equivalent can be used to generate the recombinant molecules which will direct the expression of the TGF- β 1/ β 2 product. Due to the degeneracy of the nucleotide coding sequences, other DNA sequences as depicted in FIG. 1 may be used in the practice of the present invention. Such alterations of the nucleotide sequence of FIG. 1 include deletions, additions or substitutions of different nucleotide residues resulting in a sequence that encodes the same or a functionally equivalent gene product. The gene product may contain deletions, additions or substitutions of amino acid residues within a sequence, which result in a silent change thus producing a bioactive product. Such amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity and/or the amphipathic nature of the residues involved. For example, negatively charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine and arginine; amino acids with uncharged polar head groups or nonpolar head groups having similar hydrophilicity values include the following: leucine, isoleucine, valine; glycine, alanine; asparagine, glutamine; serine, threonine; phenylalanine, tyrosine.

The nucleotide sequence for simian TGF- β 1 may be obtained from simian cell sources (Sharples et al., 1989, DNA 6:239-244). The nucleotide sequence of the chimeric TGF- β 1/ β 2 in FIG. 1 may be prepared by methods known in the art including but not limited to the use of DNA restriction enzymes, synthetic oligonucleotides, and DNA ligases. Alternatively, the coding sequence of FIG. 1 may be synthesized in whole or in part using chemical methods well known in the art.

In a specific embodiment of the invention, the coding sequence for simian TGF- β 1 was obtained from a full length cDNA clone obtained from an African green monkey cell line, BSC-40 (Sharples et al., 1987, *supra*). The coding sequence of chimeric TGF- β 1/ β 2 depicted in FIG. 1 was then derived from the simian TGF- β 1 cDNA by removing and replacing the coding sequences of amino acid residue numbers 9, 10, 11, 12, 13, 17, 19, 25 and 26 of the mature TGF- β 1 molecule with the coding sequences for amino acid residue numbers 9, 10, 11, 12, 13, 17, 19, 25 and 26 of the mature TGF- β 2 molecule (Madisen et al., 1988, DNA 7:1-8) using gene construction techniques.

5.2. CONSTRUCTION OF EXPRESSION VECTORS CONTAINING THE CHIMERIC TGF- β 1/ β 2 CODING SEQUENCE

In order to express biologically active, mature TGF- β 1/ β 2, an expression vector/host system should be chosen which provides not only for high levels of transcription and translation but for the correct processing of the gene product. This is especially important when employing the entire coding sequence of the chimeric TGF- β 1/ β 2 precursor in the expression constructs because, like TGF- β 1 and TGF- β 2, the mature chimeric TGF- β 1/ β 2 is believed to be released from a precursor molecule or complex of molecules via cellular processing events. In addition, an expression/host cell system which provides for secretion of the product may be desirable.

In particular, it appears that mature TGF- β 1/ β 2 is a disulfide linked homodimer of 112 amino acids per subunit formed by cellular processing events believed to be similar to those which form mature TGF- β 1 and TGF- β 2. The TGF- β 1/ β 2 precursor has three potential N-glycosylation sites in its pro domain (Sharples et al., 1987, DNA 6:239-244). Studies involving TGF- β 1 have determined that N-glycosylation and phosphorylation in the pro domain of TGF- β 1 occurs in

transfected CHO cells, implicating an important functional role for the precursor in the cellular synthesis and release or secretion of the mature molecule (Brunner et al., 1988, Mol. Cell. Biol. 8:2229-2232). The presence of mannose-6-phosphate in the TGF- β 1 precursor also supports the hypothesis that the precursor has independent functional activity (Purchio et al., 1988, J. Biol. Chem. 263:14211-14215). Since the chimeric TGF- β 1/ β 2 precursor contains the simian TGF- β 1 pro domain, applicants believe it likely that the TGF- β 1/ β 2 precursor is functionally active and important to the correct processing of the mature TGF- β 1/ β 2 molecule. Thus, the ability of a host cell used in the expression system to correctly express and process chimeric TGF- β 1/ β 2 is important to the production of a mature, bioactive product.

In a specific embodiment described herein, mature bioactive TGF- β 1/ β 2 is successfully produced using simian virus 40 (SV40) expression control elements in a Chinese Hamster Ovary (CHO) host cell system. However, a variety of other animal host/expression vector systems (i.e., vectors which contain the necessary elements for directing the replication, transcription and translation of the TGF- β 1/ β 2 coding sequence in an appropriate host cell) may be utilized equally well by the skilled artisan. These include, but are not limited to, virus expression vector/mammalian host cell systems (e.g., cytomegalovirus, vaccinia virus, adenovirus, and the like); insect virus expression vector/insect cell systems (e.g., baculovirus); or nonviral promoter expression systems derived from the genomes of mammalian cells (e.g., the mouse metallothionein promoter).

The expression elements of these vectors vary in their strength and specificities. Depending on the host/vector system utilized, any one of a number of suitable transcription and translation elements may be used. For instance, when cloning in mammalian cell systems, promoters isolated from the genome of mammalian cells, (e.g. mouse

metallothionein promoter) or from viruses that grow in these cells, (e.g. vaccinia virus 7.5K promoter) may be used. Promoters produced by recombinant DNA or synthetic techniques may also be used to provide for transcription of the inserted sequences.

Specific initiation signals are also required for sufficient translation of inserted protein coding sequences. These signals include the ATG initiation codon and adjacent sequences. For example, in cases where only a portion of the TGF- β 1/ β 2 coding sequence is inserted, exogenous translational control signals, including the ATG initiation codon must be provided. Furthermore, the initiation codon must be in phase with the reading frame of the TGF- β 1/ β 2 coding sequences to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of transcription attenuation sequences, enhancer elements, and the like.

Any of the methods previously described for the insertion of DNA fragments into a vector may be used to construct expression vectors containing the TGF- β 1/ β 2 coding sequence and appropriate transcriptional/translational control signals. These methods may include in vitro recombinant DNA techniques, synthetic techniques and in vivo recombinations (genetic recombination).

In cases where an adenovirus is used as an expression vector, the TGF- β 1/ β 2 coding sequence may be ligated to an adenovirus transcription/translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by in vitro or in vivo recombination. Insertion in a non-essential region of the viral genome (e.g., region E1 or E3)

will result in a recombinant virus that is viable and capable of expressing chimeric TGF- β 1/ β 2 in infected hosts.

Similarly, the vaccinia 7.5K promoter may be used.

An alternative expression system which could be used to express TGF- β 1/ β 2 is an insect system. In one such system, Autographa californica nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes. The virus grows in Spodoptera frugiperda cells. The TGF- β 1/ β 2 coding sequence may be cloned into non-essential regions (for example, the polyhedrin gene) of the virus and placed under the control of an AcNPV promoter (for example, the polyhedrin promoter). Successful insertion of the TGF- β 1/ β 2 coding sequence will result in inactivation of the polyhedrin gene and production of non-occluded recombinant virus (i.e., virus lacking the proteinaceous coat encoded by the polyhedrin gene). These recombinant viruses are then used to infect Spodoptera frugiperda cells in which the inserted gene is expressed.

In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Expression from certain promoters can be elevated in the presence of certain inducers, (e.g. zinc and cadmium ions for metallothionein promoters). Therefore, expression of the genetically engineered TGF- β 1/ β 2 may be controlled. This is important if the protein product of the cloned foreign gene is lethal to host cells. Furthermore, post-translational modifications such as glycosylation, and processing events such as proteolytic cleavage of protein products, may be important to the functionality of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins. Appropriate cell lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed.

In a specific embodiment of the invention, an expression vector containing the TGF- β 1/ β 2 coding sequence in tandem with the mouse dihydrofolate reductase gene (dhfr) under the control of SV40 regulatory sequences is constructed and used to transfect dhfr-deficient CHO cells. CHO transfectants expressing the dhfr phenotype are isolated by propagation in selective media. To increase the level of expression of TGF- β 1/ β 2, transfectants may be exposed to increasing concentrations of methotrexate in order to isolate clones transcribing amplified levels of TGF- β 1/ β 2 mRNA. TGF- β 1/ β 2 mRNA levels may be assayed at various stages of amplification by solution hybridization (Uhler et al., 1986, Proc. Natl. Acad. Sci. U.S.A. 83:1300-1304).

5.3. IDENTIFICATION OF TRANSFECTANTS EXPRESSING CHIMERIC TGF- β 1/ β 2

The host cells which contain the TGF- β 1/ β 2 coding sequence and which express the biologically active, mature product may be identified by at least four general approaches: (a) DNA-DNA hybridization; (b) the presence or absence of "marker" gene functions; (c) assessing the level of transcription as measured by the expression of TGF- β 1/ β 2 mRNA transcripts in the host cell; and (d) detection of the mature gene product as measured by immunoassay and, ultimately, by its biological activities.

In the first approach, the presence of the TGF- β 1/ β 2 coding sequence inserted in the expression vector can be detected by DNA-DNA hybridization using probes comprising nucleotide sequences that are homologous to the TGF- β 1/ β 2 coding sequence substantially as shown in FIG. 1, or portions or derivatives thereof.

In the second approach, the recombinant expression vector/host system can be identified and selected based upon the presence or absence of certain "marker" gene functions (e.g., thymidine kinase activity, resistance to antibiotics,

resistance to methotrexate, transformation phenotype, occlusion body formation in baculovirus, etc.). For example, if the TGF- β 1/ β 2 coding sequence is inserted within a marker gene sequence of the vector, recombinants containing the TGF- β 1/ β 2 coding sequence can be identified by the absence of the marker gene function. Alternatively, a marker gene can be placed in tandem with the TGF- β 1/ β 2 sequence under the control of the same or different promoter used to control the expression of the TGF- β 1/ β 2 coding sequence. Expression of the marker in response to induction or selection indicates expression of the TGF- β 1/ β 2 coding sequence.

In the third approach, transcriptional activity for the TGF- β 1/ β 2 coding region can be assessed by hybridization assays. For example, polyadenylated RNA can be isolated and analyzed by Northern blot using a probe homologous to the TGF- β 1/ β 2 coding sequence or particular portions thereof. Alternatively, total nucleic acids of the host cell may be extracted and assayed for hybridization to such probes.

In the fourth approach, the expression of the mature protein product can be assessed immunologically, for example by Western blots, immunoassays such as immunoblotting, radioimmunoprecipitation, enzyme-linked immunoassays, and the like. The ultimate test of the success of the expression system, however, involves the detection of the biologically active TGF- β 1/ β 2 gene product. Where the host cell secretes the gene product, the cell free media obtained from the cultured transfectant host cell may be assayed for TGF- β 1/ β 2 activity. Where the gene product is not secreted, cell lysates may be assayed for such activity. In either case, biological assays such as the growth inhibition assay described herein or the like may be used.

Once a clone producing high levels of mature TGF- β 1/ β 2 is identified, the clone may be expanded and the TGF- β 1/ β 2 may be purified using techniques well known in the art. Such

methods include immunoaffinity purification, chromatographic methods including high performance liquid chromatography, and the like.

5.4. IMPROVED METHOD FOR PRODUCING MATURE TGF- β 1/ β 2

In a specific embodiment of the invention, the TGF- β 1/ β 2 precursor may be modified by eliminating the amino acid cysteine at position 33 (CYS-33) of FIG. 1 and replacing it with serine. This modification may be introduced at the DNA level using site directed mutagenesis. COS cells transfected with plasmids encoding the TGF- β 1 precursor gene so modified ultimately secrete between three and five times more mature TGF- β 1 than do CHO cells expressing the unmodified precursor gene. It is believed that this modification prevents the formation of certain disulfide-linked precursor complexes in the transfected COS cells and therefore allows for the secretion of more mature TGF- β .

6. EXAMPLE: PRODUCTION OF TGF- β 1/ β 2 BY EXPRESSION IN CHINESE HAMSTER OVARY CELLS

A recombinant plasmid encoding TGF- β 1 precursor in which amino acids 9, 10, 11, 12, 13, 17, 19, 25 and 26 of the mature TGF- β 1 sequence were replaced by the corresponding amino acids of the mature TGF- β 2 sequence was constructed. Specifically, amino acid 9 of mature TGF- β 1 (serine) was replaced by arginine, amino acid number 10 (serine) was replaced by asparagine, amino acid number 11 (threonine) was replaced by valine, amino acid number 12 (glutamic acid) was replaced by glutamine, amino acid number 13 (lysine) was replaced by aspartic acid, amino acid number 17 (valine) was replaced by leucine, amino acid number 19 (glutamine) was replaced by proline, amino acid number 25 (arginine) was replaced by lysine and amino acid number 26 (lysine) was

replaced by arginine. The construct was used to transfect CHO cells. Transfectants which produced and secreted a mature, bioactive, chimeric TGF- β 1/ β 2 were isolated.

6.1. MATERIALS AND METHODS

6.1.1. DNA TRANSFECTIONS

Approximately 24 hours after seeding 10^6 dhfr-deficient CHO cells onto 100 mm dishes, the cultures were transfected with 1 μ g of NdeI linearized p5 β /dhfr plasmid and 19 μ g of calf thymus DNA as carrier as a calcium phosphate precipitate (Wigler, M., et al., 1979, Proc. Natl. Acad. Sci. U.S.A. 76:1373-1376). Briefly, 20 μ g of plasmid plus carrier DNA was added to 1 ml of 250 mM sterile CaCl_2 . The DNA solution (1 ml) was added dropwise to a 1 ml portion of 2X HEPES solution (280 mM NaCl, 50 mM HEPES, 1.5 mM sodium phosphate, pH 7.1) while bubbling and the mixture was allowed to sit on ice for 30 minutes. The precipitate was then dispersed dropwise over the cells containing 10 ml of F12 media (Gibco). After incubation at 37°C for 4 hours, the media was removed and replaced with 10 ml of F12 media containing 25% glycerol for 90 seconds at room temperature. Cells were rinsed once with 20 ml of F12 media and incubated in the nonselective F12 media (20 ml) for an additional 48 hours. Selection for dhfr expressing transfectants was accomplished by replacing the media with DMEM supplemented with 10% dialyzed FBS (Gibco) and 150 μ g/ml L-proline. Colonies were observed after culturing the cells 10-14 days in the selection media.

6.1.2. SELECTION OF METHOTREXATE RESISTANT CELLS

Dihydrofolate reductase (dhfr) amplified cells were derived from the primary transfectants essentially as described (Gasser, C.S. and Schimke, R.T., 1986, J. Biol. Chem. 261:6938-6946). After expansion, 10^5 cells were seeded

onto 100 mm dishes and adapted to increasing concentrations of methotrexate (100 nM; 500 nM; 2,500 nM; 10,000 nM; 20,000 nM). The initial concentration of methotrexate was 100 nM. The plate containing visible colonies was trypsinized and adapted to that concentration of methotrexate for at least two additional 1:5 cell passages. Cells (10^5) were then seeded onto 100 mm dishes in the next highest concentration of methotrexate. The dish containing visible colonies was again trypsinized and adapted in the methotrexate containing medium. Cells were frozen back at various stages of amplification in media containing 40% FBS, 10% dimethyl sulfoxide and 50% DMEM. Methotrexate was not included in the freezing media.

6.1.3. GROWTH INHIBITION ASSAY

Mink lung epithelial cells, Mv 1 Lu (Accession Number CCL-64, American Type Culture Collection), which are extremely sensitive to TGF- β were utilized for the growth inhibition assay. The assay was performed using the thymidine analog 5'-[125 I]-iodo-2'-deoxyuridine (125 IdU) to assess DNA synthesis. One unit of activity was defined as the amount required to inhibit 50% incorporation of 125 IdU compared to untreated CCL-64 cells.

To assay transfected cells for secretion of active TGF- β 1/ β 2, serum free supernatants were collected from one 24 hour collection on confluent cultures of cells and dialyzed extensively against 0.2 M acetic acid. Samples were diluted into sterile complete culture medium for assays.

6.1.4. PEPTIDE SYNTHESIS AND PRODUCTION OF ANTIBODIES

Peptides were synthesized by solid phase techniques on a Beckman 990 instrument, and cleaved from the resin as previously described (Gentry, L.E., et al., 1983, J. Biol. Chem. 258:11219-11228; Gentry, L.E. and Lawton, A., 1986, Virology 152:421-431). Purification was accomplished by

preparative high performance liquid chromatography. The composition of the peptides was confirmed by amino acid analysis.

Synthetic peptides were conjugated to bovine gamma-globulin through the cysteine residue. Coupling reactions were performed essentially as described (Gentry and Lawton, 1986, *supra*). The efficiencies of peptide conjugations ranged from 8 to 26 molecules of peptide covalently attached per molecule of gamma-globulin.

New Zealand white rabbits were primed at three to six sites by combined subcutaneous and intradermal inoculations with the peptide conjugates (100 ug equivalents of peptide) emulsified in Freund's complete adjuvant. Booster inoculations were administered at 2-3 week intervals. Bleedings were taken 7-14 days following the boosts.

Anti-peptide antibodies directed toward peptide sequences within the TGF- β 1 molecule were generated in rabbits using synthetic peptides as immunogens (Gentry et al., 1987, *Mol. Cell. Biol.* 7:3418-3427). One of the antibodies (anti-TGF- β 1₃₆₉₋₃₈₁) was directed toward epitopes present within the mature form of the TGF- β growth factor. The other two antibodies (anti-TGF- β 1₈₁₋₉₄ and anti-TGF- β 1₂₂₅₋₂₃₆) are precursor-specific and are directed toward peptide sequences present only within the precursor molecule of TGF- β 1.

6.1.5. IMMUNOBLOTTING

Proteins were fractionated on 7.5%-17.5% gradient SDS-polyacrylamide gels and transferred to unmodified nitrocellulose (0.45 μ m; Schleicher and Schuell) for 1 hour at 24 volts at 4°C (Burnette, W.N., 1981, *Anal. Biochem.* 112:195-203). Excess binding capacity of the nitrocellulose was blocked by incubation with 2.5% BLOTTO (Johnson, D.A., et al., 1984, *Gene Anal. Techn.* 1:3-8) in phosphate-buffered saline (PBS) containing 0.2% NP-40. Rabbit anti-serum

diluted 1:75 in 2.5% BLOTTO was incubated with the blocked nitrocellulose sheets for 2 hours at room temperature. After washing away excess antibody by five 5-minute washes in 2.5% BLOTTO, the nitrocellulose sheets were incubated with alkaline phosphatase-conjugated Protein A diluted 1:500 in 2.5% BLOTTO. Following a two hour incubation, the nitrocellulose sheets were washed 5 times in PBS (5 minute washes) containing 0.2% NP-40 and developed (Leary et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:4045-4049).

6.1.6. CONSTRUCTION OF PLASMID PROGRAMMING THE SYNTHESIS OF TGF- β 1/ β 2

The plasmid programming the synthesis of the chimeric TGF- β 1/ β 2 protein, p5 β /dhfr, was constructed as follows. pAc β TGF-1, a baculovirus vector derived from pAc373 (Miyamoto et al., 1985, Cell. Biol. 5:2860-2865; Madisen et al., 1987, Virology 158:248-250), which contains the 1.4 Kb PstI-EcoRI coding sequence of TGF- β 1 (Sharples et al., 1987, DNA 6:239-244) cloned into the PstI-EcoRI site of pAc611 (Miyamoto et al., 1985, Cell. Biol. 5:2860-2865; Madisen et al., 1987, Virology 158:248-250), was digested with BamHI and EcoRI and the 375 bp fragment of the TGF- β 1 coding sequence was isolated (Fragment 1). pSV2- β TGF (Gentry et al., 1987, Mol. Cell. Biol. 7:3418-3427) was digested with ApaI and EcoRI and the 3.5 Kb fragment was isolated (Fragment 2).

Complementary synthetic oligonucleotides having the sequences shown below were synthesized on an Applied Biosystems Oligonucleotide Synthesizer and purified from an acrylamide gel. Phosphates were added with T4 kinase and equimolar amounts of the kinased oligonucleotides were annealed. The annealed double stranded synthetic DNA was then ligated to fragments '1' and '2' described above. The ligation mixture was used to transform E. coli and 5 β pSV2(Hpa⁻Eco⁺) was isolated.

5' - CAA CAT CTG CAA AGC TCC CGG CAC CGC CGA GCC
CTG GAC ACC AAC TAC TGC TTC AGA AAT GTG CAG
GAT AAT TGC TGC CTA CGT CCG CTT TAC ATT GAT
TTC AAG AGG GAT CTA GGG TGG AAA TG - 3'

5' GAT CCA TTT CCA CCC TAG ATC CCT CTT GAA ATC
AAT GTA AAG CGG ACG TAG GCA GCA ATT ATC CTG
CAC ATT TCT GAA GCA GTA GTT GGT GTC CAG GGC
TCG GCG GTG CCG GGA GCT TTG CAG ATG TTG GGC C - 3'

5 β pSV2(Hpa⁻Eco⁺) was digested with EcoRI, filled in with Klenow enzyme, digested with HindIII and the 1.4 Kb fragment containing the chimeric TGF- β 1/ β 2 coding sequence was isolated (Fragment 3). 5 β pSV2 was constructed by ligating Fragment 3 into pSV2,neo which had previously been digested with HindIII and HpaI to eliminate the neo gene.

5 β pSV2 was digested with EcoRI, filled in with Klenow enzyme, digested with NdeI and the 2.6 Kb NdeI-EcoRI (blunt) fragment was isolated and ligated to pSV2/dhfr (Gentry et al., 1987, Mol. Cell. Biol. 7:3718-3727) which had been digested with NdeI and PvuII. The ligation mixture was used to transform E. coli and p5 β /dhfr was isolated. The nucleotide and deduced amino acid sequences of the chimeric TGF- β 1/ β 2 molecule encoded by p5 β /dhfr are shown in FIG. 1.

6.2. EXPRESSION OF TGF- β 1/ β 2 IN CHO CELLS

p5 β /dhfr was transfected into CHO cells and single clones were amplified with methotrexate as described in Section 6.1., supra. One such amplified clone, CHO-5 β 41,2.5, was chosen for further characterization.

CHO-5 β 41,2.5 cells were grown to confluence in 2.5 μ M methotrexate. Media was replaced with serum free media and, after 24 hr, was collected and dialyzed for 48 hr against 0.2M acetic acid. Dialyzed, conditioned supernatants were assayed for bioactivity by inhibition of DNA synthesis of

CCL-64 cells as described in Section 6.1.3., supra. CHO-5 β 41,2.5 cells secrete approximately 1 mg/L of bioactive chimeric TGF- β 1/ β 2 (FIG. 2).

TGF- β related proteins secreted by these cells were analyzed by immunoblotting using anti-peptide antibodies directed against mature TGF- β 1 as described in Section 6.1.5., supra. FIG. 3 shows that CHO-5 β 41,2.5 cells secrete immunoreactive proteins migrating at 90 to 100 kilodaltons and at 24 kilodaltons when analyzed on SDS-PAGE under nonreducing conditions (FIG. 3, lane 1). The 24 kilodalton band represents the mature TGF- β 1/ β 2 dimer and the 90 to 100 kilodalton protein probably represents mature TGF- β 1/ β 2 disulfide-bonded to precursor sequences (Gentry et al., 1987, Mol. Cell. Biol. 7:3418-3427).

Under reducing conditions (FIG. 3, lane 2), the majority of the proteins migrate at 12 kilodaltons, representing the mature TGF- β 1/ β 2 monomer. Note the lack of immunoreactive material in the 45 to 55 kilodalton range observed in a similar analysis of recombinant proteins expressed in CHO cells transfected with plasmids encoding the simian TGF- β 1 gene (Gentry et al., 1987, Mol. Cell. Biol. 7:3418-3427) suggesting that chimeric TGF- β 1/ β 2 is proteolytically processed more efficiently than its parent molecule TGF- β 1. In addition, CHO-5 β 41,2.5 cells secrete about 2.5 times more bioactive mature product than do CHO cells expressing TGF- β 1 (Gentry et al., 1987, supra). Although the basis for these observations is presently unknown, the secondary structure of the chimeric TGF- β 1/ β 2 precursor may significantly differ from the secondary structure of TGF- β 1, which secondary structure renders the chimeric TGF- β 1/ β 2 subject to molecular processing events of a different intensity or nature. For example, the TGF- β 1/ β 2 precursor may be a more favorable substrate for the factors involved in TGF- β processing. Alternatively, the secondary

structural characteristics of TGF- β 1/ β 2 may allow it to interact with other processing factors or pathways not as accessible to TGF- β 1.

7. EXAMPLE: GROWTH MODULATION OF VASCULAR
ENDOTHELIAL CELLS BY HYBRID TGF-5 β

Using three distinct bioassays which previously indicated that differential responses are induced by TGF- β 1 and TGF- β 2 (Merwin et al., 1991, Am. J. Path. 138(1):37-51), the biological effects induced by recombinant hybrid TGF- β 1/ β 2 (TGF-5 β) on the proliferation, migration and angiogenesis of large vessel endothelial, smooth muscle and microvascular endothelial cells were examined.

7.1. MATERIALS AND METHODS

7.1.1. CELL CULTURE

Bovine aortic smooth muscle cells (BASMCs) were grown from explants of bovine aorta in complete DME supplemented with 10% FCS as described in Kocher and Madri, 1989, In Vitro Cell. & Dev. Biol. 25:424-34. After several days in culture, BASMCs migrate out from the medial explants. Following the development of confluency, the cells were trypsinized, passaged and utilized in the growth inhibitory assays described, infra. These α -smooth muscle, actin positive cells formed the hill-and-valley pattern typical of cultured smooth muscle cells and were used between passage 2 and 5.

Capillary endothelial cells were isolated and cultured from rat epididymal fat pads as described in Madri and Williams, 1983, J. Cell Biol. 97:153-65, and are hereinafter termed "RFCs". Bovine calf aortic endothelial cells (BAECs) were isolated and cultured as described in Madri and Furthmayr, 1980, Hum. Path. 11:353-66. Three-dimensional RFC cultures were composed of acid soluble calf dermis collagen type I and prepared using the method of Madri et al., 1988,

J. Cell Biol. 106:1375-84. To address the possibility of differences due to the use of two species, proliferative, migratory and angiogenic studies were also performed using bovine adrenal cortex microvascular endothelial cells; the results were equivalent to those obtained with RFCs.

7.1.2. TGF- β FACTORS

Recombinant hybrid TGF- β 1/ β 2 was produced as described in Section 6, et seq, supra. TGF- β 1 and TGF- β 2 were prepared, respectively, as described in Assoian et al., 1983, J. Biol. Chem. 258:7155-60, and in Cheifetz et al., 1987, Cell 48:409-15, and were obtained from Drs. A. Roberts and M. Sporn at the Laboratory of Chemoprevention, National Cancer Institute, Bethesda, MD.

7.1.3. MATRIX COMPONENTS

Tissue culture dishes and flasks were coated with purified bovine type I collagen (Madri and Furthmayr, 1980, Hum. Path. 11:353-66) at a concentration of 12.5 μ g/ml.

7.1.4. PROLIFERATION INHIBITION ASSAY

Collagen type I-coated dishes were washed in PBS before the addition of cell suspensions (1.4×10^4 cells/dish). After 6 h, the "initial" number of cells on the substrate was determined. At this time point, fresh medium with or without TGF- β s was added to the cultures. Medium and TGF- β s were replaced once again on day 3. Cell numbers were determined by lifting the cells off the culture dishes with trypsin/EDTA and counting quadruplicate samples using a Coulter counter (Coulter Electronics, Inc., Hialeah, FL). The mean number of cells per dish for each TGF- β addition was then calculated.

7.1.5. CELL MIGRATION ASSAYS

Stimulus for cell migration was accomplished by releasing confluent cultures from contact inhibition using a stainless steel "fence" device as previously described (Pratt et al., 1984, Am. J. Path. 117:349-54). Once confluency had been achieved in the center wells of the fences (~6 h), migration was induced by removing the fences, and cells were thereafter observed migrating outward in a radial fashion. Cultures were fed once again on day 3 with control or TGF- β -supplemented media. After 6 days, the cultures were washed with PBS, fixed with 10% neutral buffered formalin and stained with Harris hematoxylin. Net increase in surface area covered was assessed using an overhead projector, and areas were calculated utilizing a computerized graphics tablet and "MacMeasure" software (Yale Shareware, Yale University, New Haven, CT).

7.2. RESULTS

The majority of published reports indicate that the TGF- β 1 and TGF- β 2 isoforms are equipotent in vitro. However, recent reports indicate that these isoforms are not functionally interchangeable, since they elicit dissimilar effects and exhibit different spatial and temporal localizations (See, for example, Ohto et al., 1987, Nature 329:539-41; Rosa et al., 1988, Science 239:783-85; and Danielpour et al., 1989, J. Cell Physiol. 138:79-86). TGF- β 1 is reportedly more effective than TGF- β 2 in inhibiting DNA synthesis in endothelial cells (Jennings, et al., 1988, J. Cell. Physiol. 137:167-72).

A recent report establishes that TGF- β 1 and TGF- β 2 isoforms elicit differential responses in proliferation assays using three vascular cell types (Merwin et al., 1991, Am J. Path. 138(1):37-51). Therefore, investigating the effects of TGF- β on endothelial cells seemed ideal for determining whether the TGF- β 1/TGF- β 2 hybrid (TGF- β 5) would

evoke responses similar to TGF- β 1 or to TGF- β 2. The results indicated that TGF-5 β mimicked TGF- β 1 in all three proliferation assays examined, while TGF- β 2 displayed unique properties.

Both TGF- β 1 and TGF-5 β inhibited BAEC proliferation by more than 70% (+/-1.5%) at the optimum concentration of 0.5 ng/ml (FIG. 4A). In contrast, TGF- β 2 was only able to evoke inhibition of about 20% (+/- 2.5%). When using BASMCs, all three growth factors exhibited similar inhibitory activities (FIG. 4B). Microvascular endothelial cells were growth inhibited as much as 70% (+/- 4.0%) by both TGF- β 1 and TGF-5 β at concentrations of 0.5 ng/ml (FIG. 4C). At equivalent concentration, however, TGF- β 2 was only able to mount a 37% (+/- 8%) inhibition; however, with a 10-fold increase in concentration, TGF- β 2 was able to evoke a similar response.

Similarly, hybrid TGF-5 β exerted a cell migration response equivalent to that induced by TGF- β 1. The results presented in FIG. 5 show that BAECs are inhibited by both TGF-5 β and TGF- β 1, while BASMCs are growth-stimulated by both factors. In contrast, TGF- β 2 had no effect on the migration of either cell type.

The above results may help to explain vascular response to injury. Upon denudation (i.e., following angioplasty) of the endothelial barrier, platelets adhere and aggregate to the newly exposed subendothelial extracellular matrix and release a variety of molecules. In this regard, one of the major factors released by platelets is TGF- β 1. With a marked increase in the local concentration of TGF- β 1, endothelial cells may be inhibited from migrating to affect a rapid re-endothelialization of the injury while at the same time, smooth muscle cell migration toward an increased factor gradient may be stimulated, thereby establishing an ideal opportunity for perpetuating the expansion of the medial compartment of the vessel wall and continued occlusion of the vascular lumen.

While angiogenesis is an important and necessary response during embryogenesis and tissue repair, it is an undesirable phenomenon in the context of solid tumor growth. Therefore, understanding the enhancement of neovascularization is of paramount concern. To investigate the TGF- β -induced angiogenic response in microvascular endothelial cells, RFCs were grown in three-dimensional collagen cultures for 4 days, either in the presence or absence of TGF- β isoforms. All three isoforms were able to stimulate RFCs to mount a neovascularization response. This endothelial "activation" was previously defined by extensive EM analysis (Merwin et al., 1990, J. Cell. Physiol. 142:117-29) which revealed the formation of branching tubular structures, cell-cell junctional complexes, metabolic stimulation (increased numbers of ribosomes and engorged ER), cellular polarity, deposition and organization of extracellular matrix proteins and lumen formation.

FIG. 6A shows control cultures with no exogenous growth factor. Cells remain independent of one another and lack tubular structures, while treatment with TGF- β 1 (FIG. 6B) reveals complex, tube-like formations. TGF- β 2 (FIG. 6C) was able to initiate a modest angiogenic response. However, as with RFC proliferation studies, an increase in TGF- β 2 concentration from 0.5 to 5.0 ng/ml was necessary for the establishment of complex tubular structures. TGF- β 5 mimicked TGF-1, with tube formation initiated with as little as 0.05 ng/ml (FIG. 6D), and optimal neovascularization achieved at a concentration of 0.5 ng/ml (FIG. 6E). A slight inhibition of the angiogenic response was noted at the highest concentration used, 5.0 ng/ml (FIG. 6F).

In summary, TGF- β 5 mimics TGF- β 1 in inhibiting proliferation of low density monolayer micro and large vessel endothelial cell cultures by about 70% and by about 45% on BASMCs. In contrast, an equivalent concentration of TGF- β 2 is ineffective with BAECs and equivalent on BASMCs.

Moreover, a 10-fold increase in TGF- β 2 concentration is required to elicit a similar response with RFCs. When using a culture which begins with a tight, confluent monolayer, response induced by the isoforms differ. In particular, both TGF- β 1 and TGF- β 2 induce equivalent levels of BASMC migration enhancement and BAEC migration inhibition, while TGF- β 2 does not influence the migration of either cell type. Finally, in microvascular endothelial cells which respond to injury in vivo by neovascularization, all three TGF- β s were able to stimulate angiogenesis in vitro. However, a 10-fold increase in TGF- β 2 concentration was necessary to match the level of response induced by TGF- β 1 and TGF- β 5.

8. DEPOSIT OF MICROORGANISMS

The following transfectant has been deposited with the American Type Culture Collection, Rockville, MD, and has been assigned the listed accession number.

| <u>Transfectant</u> | <u>Plasmid</u> | <u>Accession No.</u> |
|---------------------------|------------------|----------------------|
| CHO-5 β 41,2.5 CL 5 | p5 β /dhfr | CRL 9959 |


The present invention is not to be limited in scope by the cell line deposited or the embodiments disclosed herein which are intended as single illustrations of one aspect of the invention and any which are functionally equivalent are within the scope of the invention. Indeed, various modifications of the invention, in addition to those shown and described herein, will become apparent to those skilled in the art from the foregoing description. Such modifications are intended to fall within the scope of the appended claims.

It is also to be understood that all base pair and amino acid residue numbers and sizes given for nucleotides and peptides are approximate and used for the purposes of description.

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International Application No: PCT/

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| MICROORGANISMS | |
|--|--|
| Optional Sheet in connection with the microorganism referred to on page <u>29</u> , lines of the description * | |
| A. IDENTIFICATION OF DEPOSIT * Further deposits are identified on an additional sheet <input type="checkbox"/> * | |
| Name of depository institution * American Type Culture Collection | |
| Address of depository institution (including postal code and country) * 12301 Parklawn Drive Rockville, MD 20852 US | |
| Date of deposit * January 11, 1989 | Accession Number * CRL 9959 |
| B. ADDITIONAL INDICATIONS * (leave blank if not applicable). This information is continued on a separate attached sheet <input type="checkbox"/> | |
| | |
| C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE * (if the indications are not all designated States) | |
| | |
| D. SEPARATE FURNISHING OF INDICATIONS * (leave blank if not applicable) The indications listed below will be submitted to the International Bureau later * (Specify the general nature of the indications e.g., "Accession Number of Deposit") | |
| | |
| E. <input checked="" type="checkbox"/> This sheet was received with the International application when filed (to be checked by the receiving Office) | |
| <div style="text-align: center;"> (Authorized Officer)</div> | |
| <input type="checkbox"/> The date of receipt (from the applicant) by the International Bureau * | |
| was | <div style="text-align: center;"> (Authorized Officer)</div> |

Form PCT/RO/134 (January 1981)

WHAT IS CLAIMED IS:

1. A pharmaceutical composition comprising chimeric transforming growth factor $\beta 1/\beta 2$ for use in inhibiting the proliferation of vascular endothelial cells.
2. A pharmaceutical composition comprising chimeric transforming growth factor $\beta 1/\beta 2$ for use in inducing smooth muscle cell migration.
3. A pharmaceutical composition comprising an anti-chimeric transforming growth factor $\beta 1/\beta 2$ antibody for use in inhibiting microvascular cell neovascularization.

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| | | | |
|-----------------|------|---|-----------------------|
| Simian Human | -261 | AGGGATCTGTGGCAGGTCGGAGA--AAAGA CTCC...C...CCA...A..CCCT.TTC..... | -234 |
| Simian Human | | TC--CGTCTCCTGGTACCAGATCTCGCCCATCTAGGTATTTCGGTGGGATACTGAGACACCCCCGGTCCAAGCCCTCC C.ACC.AC..T.....G..... | -158 |
| Simian Human | | CCTCCACCACTGGGCCCTTCTCCCTGAGGA-CCTCAACTTTCCTCGAGGCCCTCCCTACCTTTTCCCGGGGACCCCAG.....G.....A..... | -80 |
| Simian Human | | <u>GCCCCCTGCAGGGGGGGCCCTCCCAACAACTAGCCCTGTTCGGCTCTCGGCAGTGCCTGGGGGGCGCCCTCCCCC</u>C..C..... | -1 |
| Simian Human | | Met Pro Pro Ser Gly Leu Arg Leu Leu Leu Pro Leu Leu Trp Leu Leu Val ATG CCG CCC TCC GGG CTG CCG CTG CTG CCG CTG CTA CCA CTA CTG TGG CTA CTG GTGGly Pro Leu Thr Pro Ser Arg Pro Ala Ala Gly Leu Ser Thr Cys Lys Thr Ile Asp Met Glu Leu CTG ACG CCT AGC CGG CCG GCG GCA GGA CTA TCC ACC TGC AAG ACT ATC GAC ATG GAG CTGG... .C.G... .. | 20 60 40 120 |
| Simian Human | | Val Lys Arg Lys Arg Ile Glu Thr Ile Arg Gly Ile Arg Gly Gln Ile Leu Ser Lys Leu Arg Leu Ala GTG AAG CCG AAG CCG ATC GAG ACC ATC CGC GGC CAG CAG ATC CTG TCC TCC AAG CTG CGG CTC GCCG... ..G... .. | 50 180 60 |
| Simian Human | | Ser Pro Pro Ser Gln Gly Glu Val Pro Pro Gly Pro Leu Pro Glu Ala Val Leu Ala Leu AGC CCC CCG AGC CAG GGG GAG GTG CCG CCG GGC CCG CTG CCC GAG GCC GTG CTC GCC CTGG... ..G... .. | 70 240 80 |
| Simian Human | | Tyr Asn Ser Thr Arg Asp Arg Val Ala Gly Glu Ser Ala Glu Pro Glu Pro Glu Pro Glu TAC AAC AGC ACC CGC GAC CCG GTG GCC GCG GAG AGT GCG GAG CCG GAG CCC GAA CCG GAGG... ..G... ..A... ..A... ..G... ..T... .. | 90 300 100 |

FIG.1A

| | | | | | |
|-----------------|--|-----|--|--------------------------------------|-----|
| Simian Human | Ala Asp Tyr Tyr Ala Lys Glu Val Thr Arg GCC GAC TAC TAC GCC AAG GAG GTC ACC CGC | 110 | Val Leu Met Val Glu Thr His Asn Glu Ile GTG CTA ATG GTG GAA ACC CAC AAC GAA ATC | 120 | 360 |
| Simian Human | Tyr Asp Lys Phe Lys Gln Ser Thr His Ser TAT GAC AAG TTC AAG CAG AGC ACA CAC AGC | 130 | Ile Tyr Met Phe Phe Asn Thr Ser Glu Leu ATA TAT ATG TTC TTC AAC ACA TCA GAG CTC | 140 | 420 |
| Simian Human | Arg Glu Ala Val Pro Glu Pro Val Leu Leu CGA GAA GCA GTA CCT GAA CCT GTG TTG CTC | 150 | Ser Arg Ala Glu Leu Arg Leu Leu Arg TCC CGG GCA GAG CTG CGT CTG CTG CTG | Arg --- Arg --- AGG AGG ... | 477 |
| Simian Human | Leu Lys Leu Lys Val Glu Gln His Val Glu CTC AAG TTA AAA GTG GAG CAG CAT GTG GAG | 170 | Tyr Gln Lys Tyr Ser Asn Asn Ser Trp TAC CAG AAA TAC AGC AAC AAT TCC TGG | 537 | |
| Simian Human | Arg Tyr Leu Ser Asn Arg Leu Ala Pro Ala CGA TAC CTC AGC AAC CCG CTG GCG CCC | 190 | Asp Ser Asn Ser Pro Glu Trp Leu Ser Phe Asp G... ..AAA ... | 597 | |
| Simian Human | Val Thr Gly Val Val Arg Gln Trp Leu Ser GTC ACC GGA GTT GTG CAG TGG TTG AGC CGC | 210 | Gly Glu Ile Glu Gly Phe Arg Leu GGA GGG GAA ATT GAG GGC TTT CGC CTT | 657 | |
| Simian Human | Ser Ala His Cys Ser Cys Asp Ser Lys Arg AGC GCC CAC TGC TCC TGT GAC AGC AAA | 230 | Thr Leu Gln Val Asp Ile Asn Gly Phe ACA CTG CAA GTG GAC ATC AAC AAC GGG TTC | 717 | |

FIG.1B

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| | | | | | | | | | | | | | | | | | | | | | | |
|-----------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|------|
| Simian Human | 240 | Thr | Thr | Gly | Arg | Arg | Gly | Asp | Leu | Ala | Thr | Ile | His | Gly | Met | Asn | Arg | Pro | Phe | Leu | Leu | 777 |
| | | ACT | ACC | GGC | CGC | CGA | GGT | GAC | CTG | GCC | ACA | ATT | CAT | GGC | ATG | AAC | CGG | CCT | TTC | CTG | CTT | |
| | | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | |
| | 260 | Leu | Met | Ala | Thr | Pro | Leu | Glu | Arg | Ala | Gln | His | Leu | Gln | Ser | Ser | Arg | His | Arg | Arg | Ala | 837 |
| Simian Human | | CTC | ATG | GCC | ACC | CCA | CTG | GAG | AGG | GCC | CAA | CAT | CTG | CAA | AGC | TCC | CGG | CAC | CGC | CGA | GCC | |
| | | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | |
| | 280 | Leu | Asp | Thr | Asn | Tyr | Cys | Phe | Arg | Asn | Val | Gln | Asp | Asn | Cys | Cys | Leu | Arg | Pro | Leu | Tyr | 897 |
| Simian Human | | CTG | GAC | ACC | AAC | TAC | TGC | TTC | AGA | AAT | GTG | CAG | GAT | AAT | TGC | TGC | CTA | CGT | CCG | CTT | TAC | |
| | | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | |
| | 300 | Ile | Asp | Phe | Lys | Arg | Asp | Leu | Gly | Trp | Lys | Trp | Ile | His | Glu | Pro | Lys | Gly | Tyr | His | Ala | 957 |
| Simian Human | | ATT | GAC | TTC | AAG | AGG | GAC | CTC | GCC | TGG | TGG | ATC | ATC | CAC | GAG | CCC | AAG | GGC | TAC | CAT | GCC | |
| | | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | |
| | 320 | Asn | Phe | Cys | Leu | Gly | Pro | Cys | Pro | Tyr | Ile | Trp | Ser | Leu | Asp | Thr | Gln | Tyr | Ser | Lys | Val | 1017 |
| Simian Human | | AAC | TTC | TGC | CTG | GGG | CCC | TGT | CCC | TAC | ATT | TGG | AGC | CTG | GAC | ACG | CAG | TAC | AGC | AAG | GTC | |
| | | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | |
| | 340 | Leu | Ala | Leu | Tyr | Asn | Gln | His | Asn | Pro | Gly | Ala | Ser | Ala | Ala | Pro | Cys | Cys | Val | Pro | Gln | 1077 |
| Simian Human | | CTG | GCC | CTG | TAC | AAC | CAG | CAT | AAC | CCG | GCC | GCC | TCG | GCG | GCG | CCG | TGC | TGC | GTG | CCG | CAG | |
| | | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | |

FIG.1C

| | | | |
|-----------------|-----|---|------|
| Simian Human | 360 | Ala Leu Glu Pro Leu Pro Ile Val Tyr Tyr Val Gly Arg Lys Pro Lys Val Glu Gln Leu GCG CTG GAG CCA CTG CCC ATC ATC GTG TAC TAC GTG GGC CGC AAG CCC AAG GTG GAG CAG CTGG 370 | 1137 |
| Simian Human | 380 | Ser Asn Met Ile Val Arg Ser Cys Lys Cys Ser TCC AAC ATG ATC ATC GTG CGC TCC GTC AAA TGC AGCG 390 | 1204 |
| Simian Human | | GCCCGCCCCGCCCCACCCACCCCGCTGTCTTGTCCCTTGGGGGCTGTATTAAAGGACACCCCGTGCCCCCAAGCCACC A G G A TGGGGCCCCATTAAAGA | 1283 |
| Simian Human | | | 1300 |

FIG.1D

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FIG. 2A

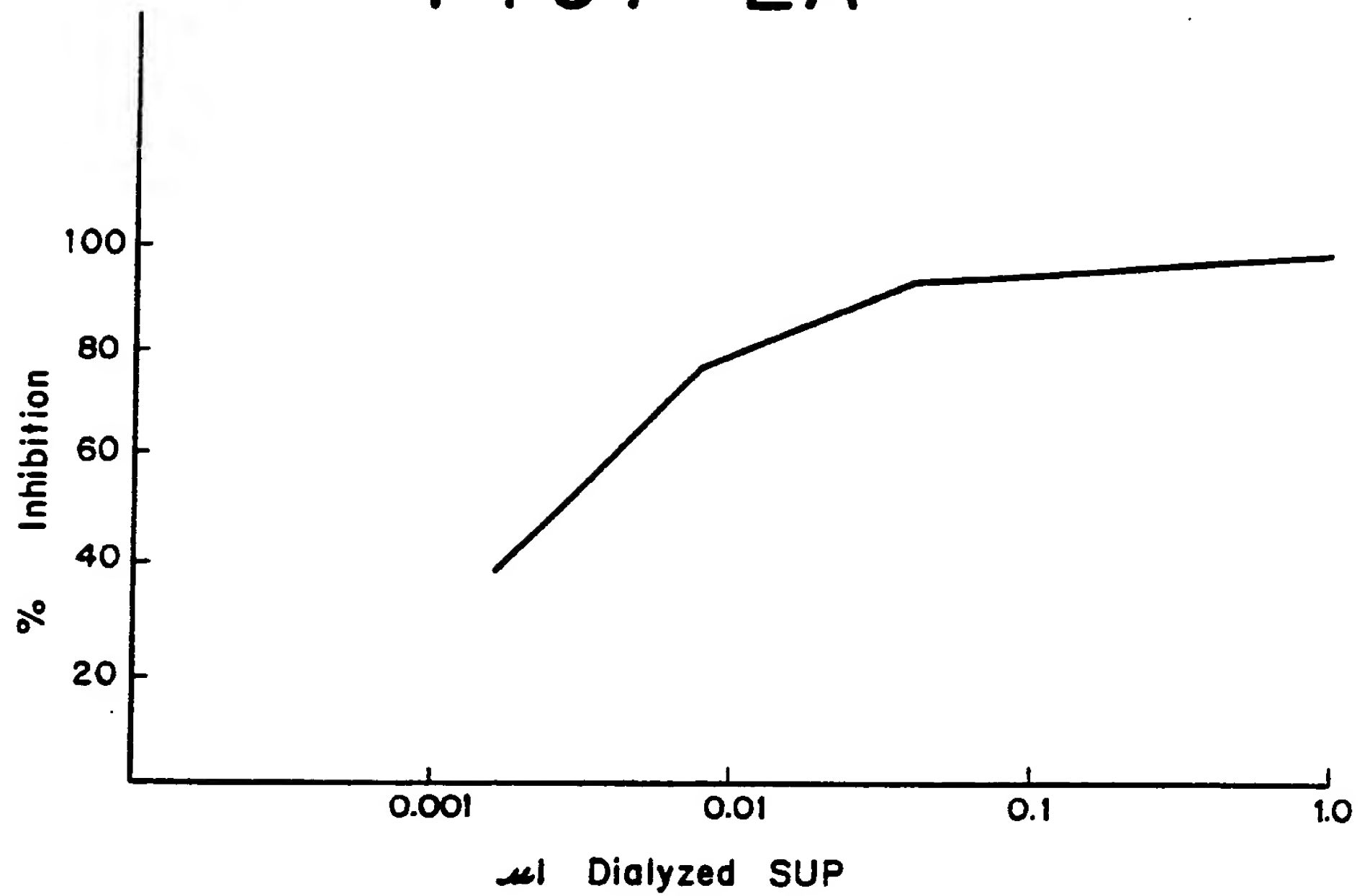
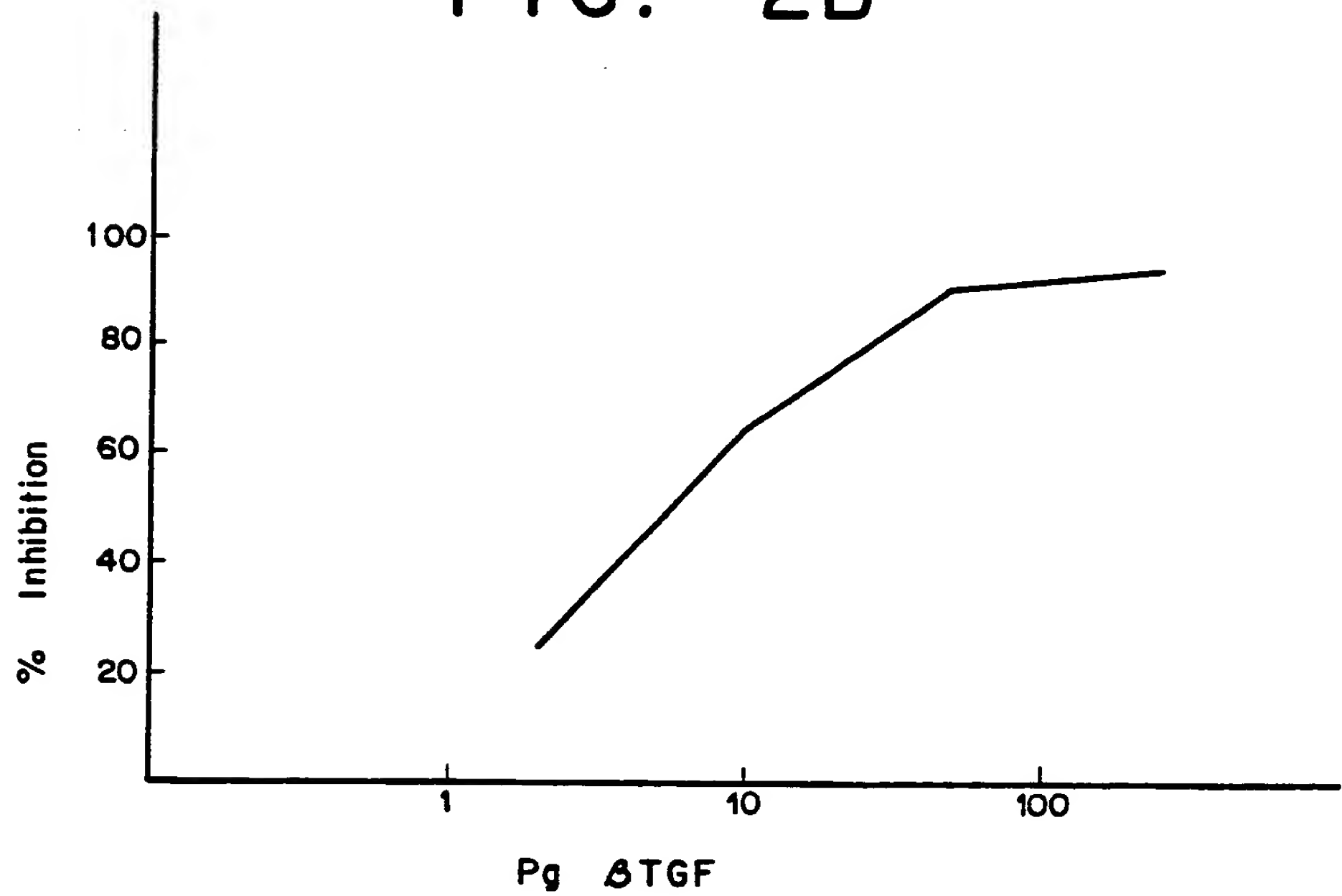
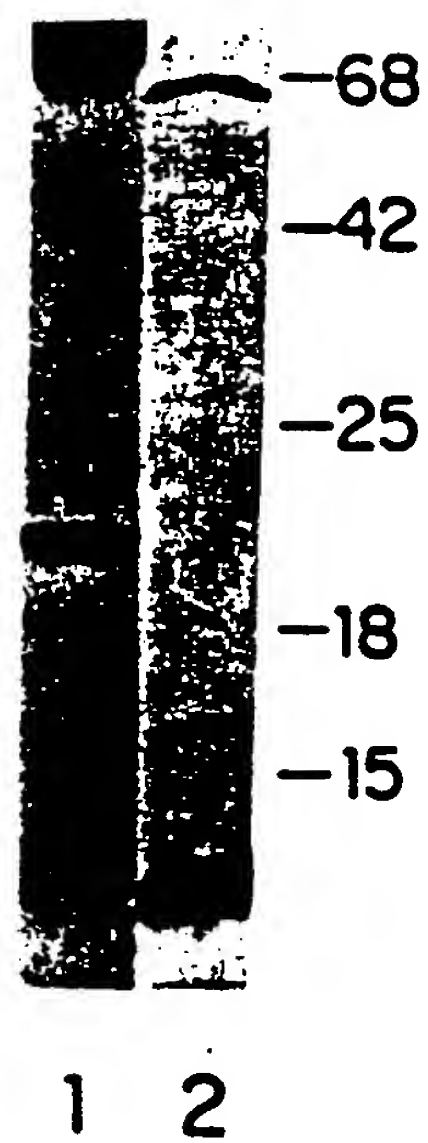


FIG. 2B



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FIG. 3



7/11
FIG. 4A

A.

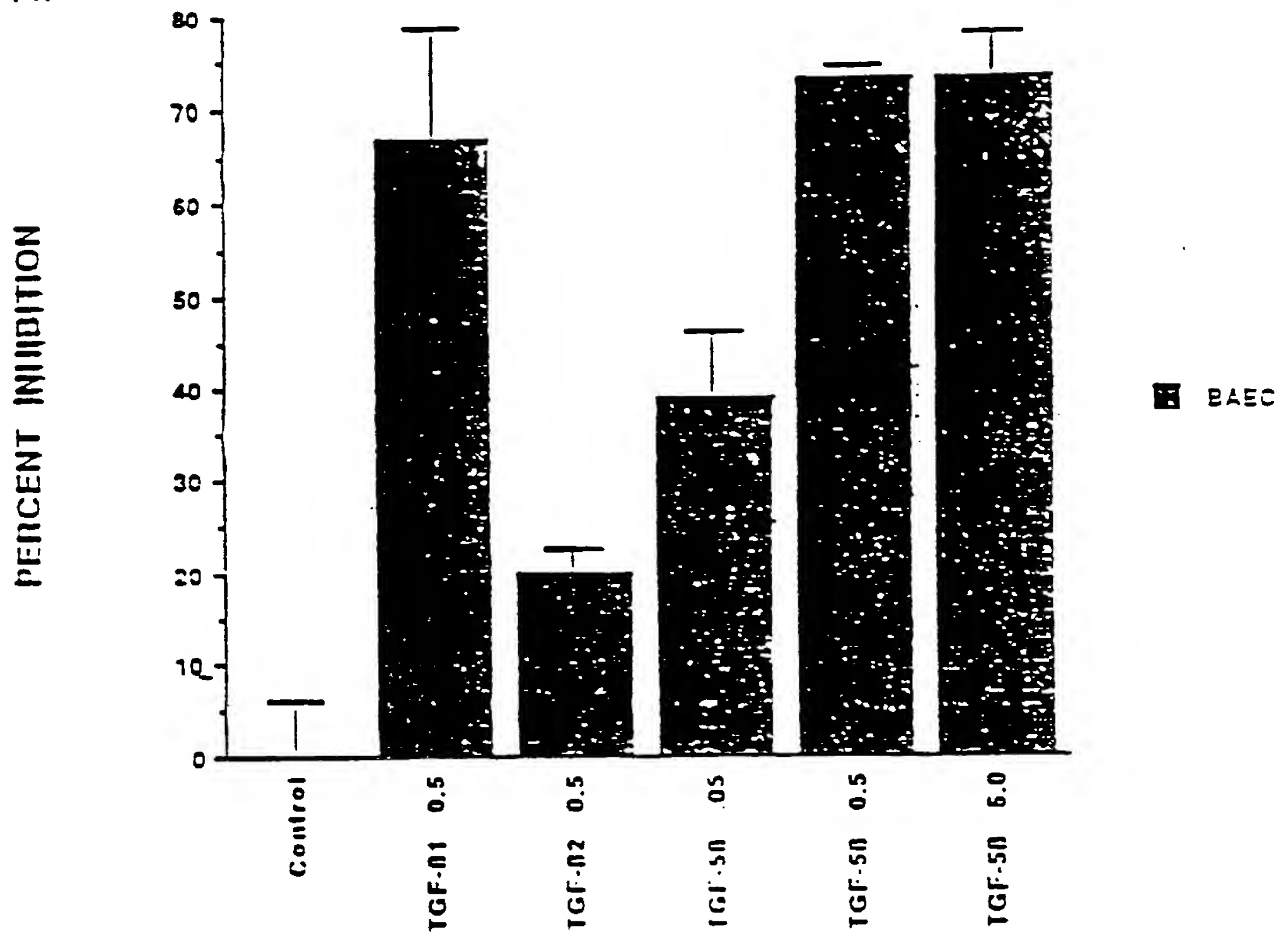


FIG. 4 B

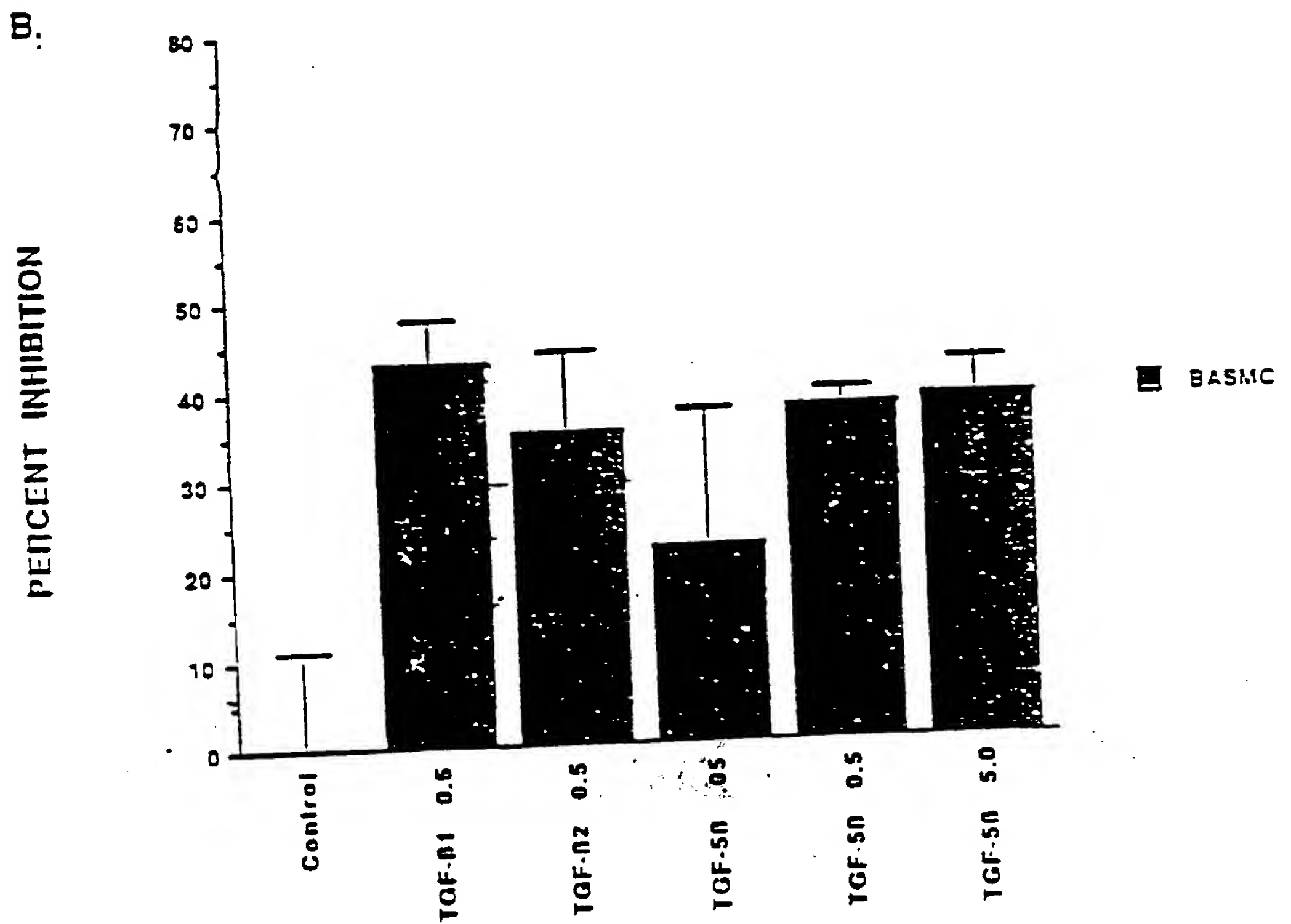


FIG. 9C

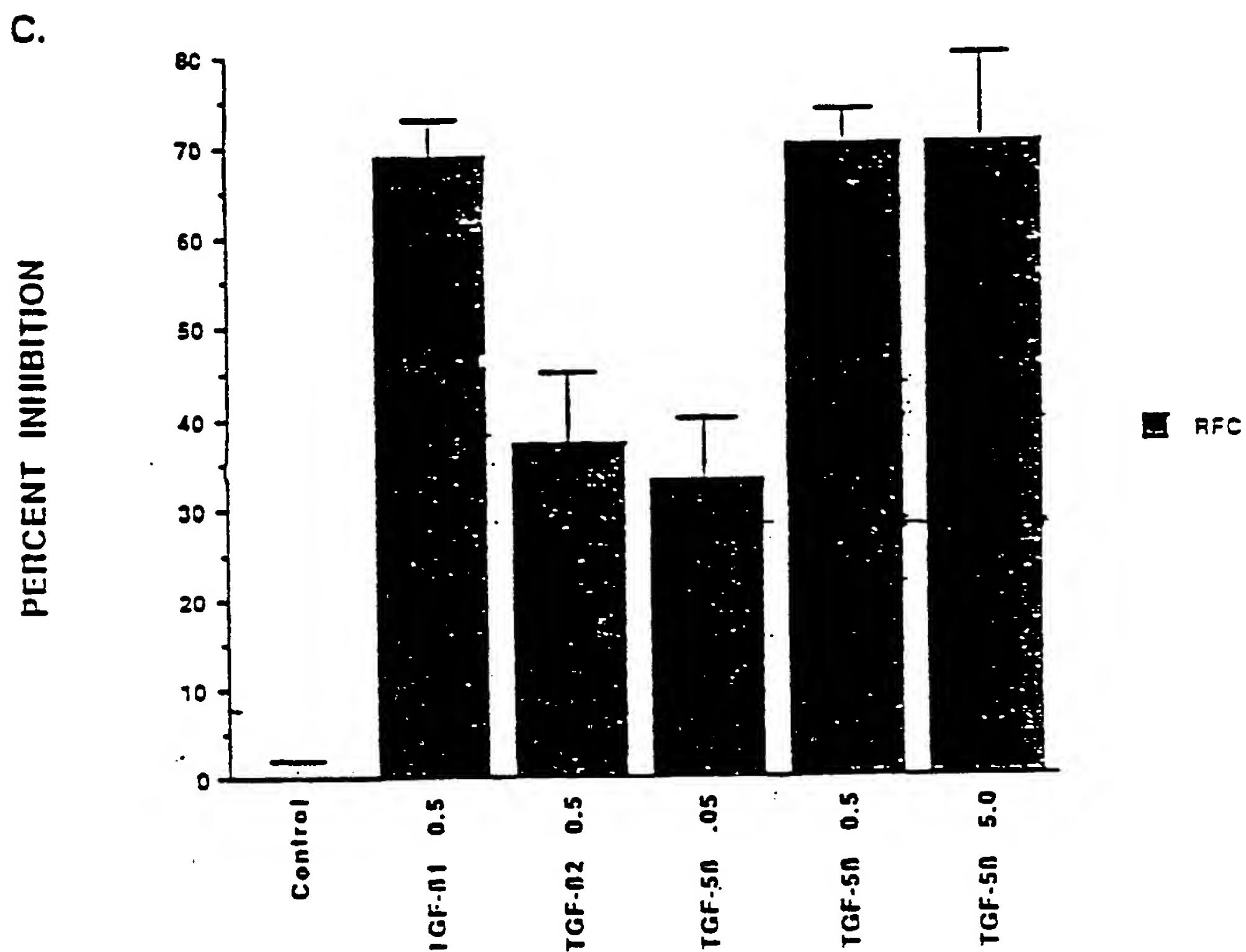


FIG. 5

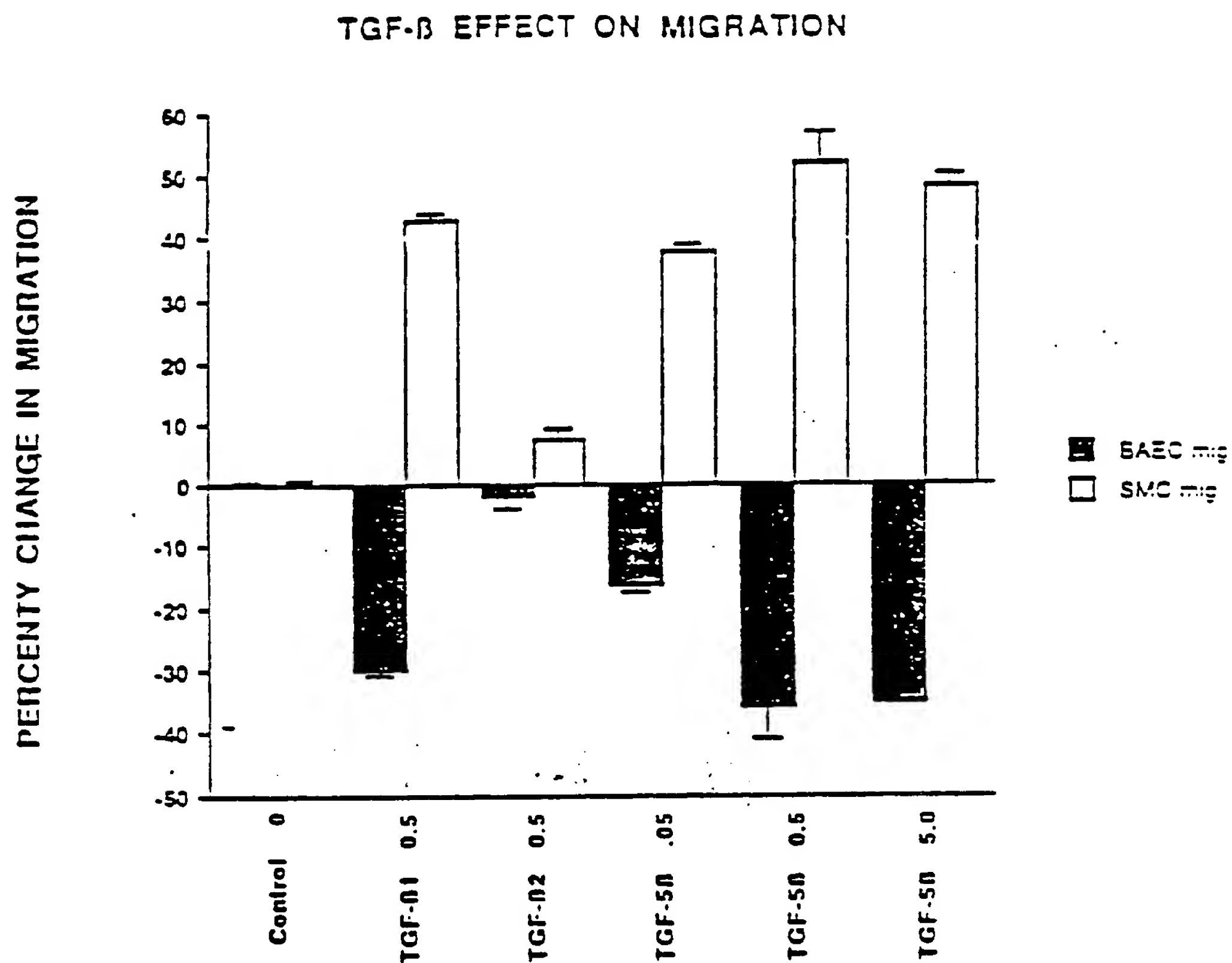
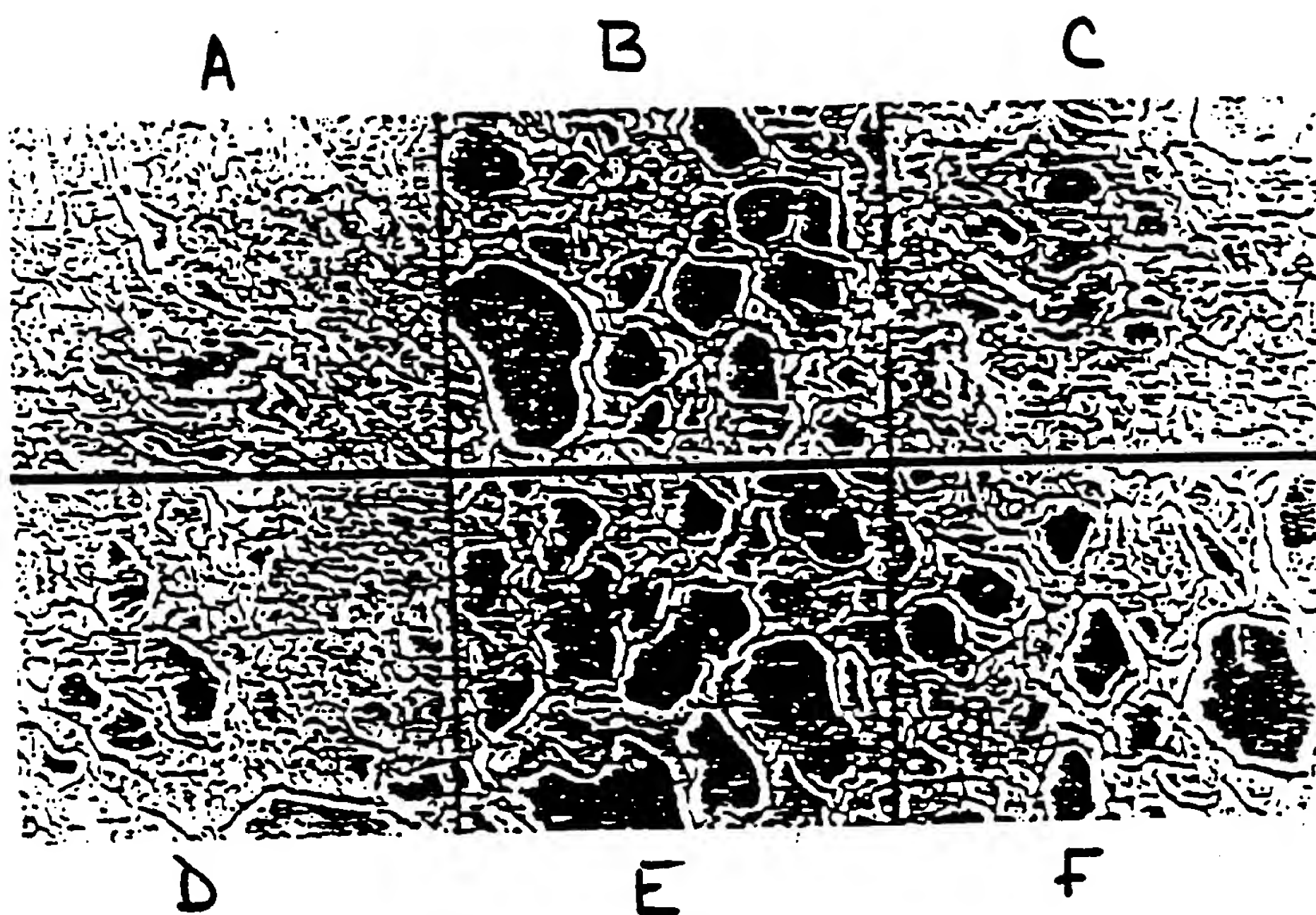


FIG. 6



INTERNATIONAL SEARCH REPORT

International Application No. PCT/US92/01993

| | | |
|--|--|--|
| I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ³ | | |
| According to International Patent Classification (IPC) or to both National Classification and IPC IPC (5): A61K 37/24; C07K 13/00 US CL : 514/12; 530/399 | | |
| II. FIELDS SEARCHED | | |
| Minimum Documentation Searched ⁴ | | |
| Classification System | Classification Symbols | |
| U.S. | 514/12; 530/399 | |
| Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched ⁵ | | |
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| III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴ | | |
| Category* | Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷ | Relevant to Claim No. ¹⁸ |
| A | US, A, 4,742,003 (Derynck, et al.) 03 MAY 1988. | 1-3 |
| A | J. BIOL. CHEM., Volume 265, issued 05 November 1990, R. Lafyatis, "Structural and Functional Characterization of the Transforming Growth Factor beta 3 Promotor. A cAMP-Responsive Element Regulates Basal and Induced Transcription," pages 19128-36. | 1-3 |
| A,E | CLINICAL RESEARCH, volume 40, APRIL 1992, A.B. Kulkarni, "Mouse Chimeras With A Disrupted Transforming Growth-Beta-1 Gene Obtained By Gene Targeting", page 295, N2. | 1-3 |
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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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| (21) International Application Number: PCT/US92/01993 (22) International Filing Date: 13 March 1992 (13.03.92) (30) Priority data: 669,171 14 March 1991 (14.03.91) US (71) Applicant: BRISTOL-MYERS SQUIBB COMPANY [US/ US]; 345 Park Avenue, New York, NY 10154 (US). (72) Inventors: PURCHIO, Anthony, F. ; 801 33rd East, Seattle, WA 98112 (US). MADISEN, Linda ; 5255 South Brigh- ton, Seattle, WA 98118 (US). MERWIN, June ; 19 Kenil- worth Drive, Clinton, CT 06413 (US). | | (74) Agent: MISROCK, S., Leslie; Pennie & Edmonds, 1155 Avenue of the Americas, New York, NY 10036 (US). (81) Designated States: AT (European patent), AU, BE (Euro- pean patent), CA, CH (European patent), DE (Euro- pean patent), DK (European patent), ES (European pa- tent), FI, FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, KR, LU (European patent), MC (European patent), NL (Eu- ropean patent), NO, SE (European patent). Published <i>With international search report.</i> |
| (54) Title: TGF- β 1/ β 2: A NOVEL CHIMERIC TRANSFORMING GROWTH FACTOR-BETA (57) Abstract The present invention relates to the production of large quantities of a novel chimeric TGF- β , termed TGF- β 1/ β 2, by eu- caryotic host cells transfected with recombinant DNA vectors containing the TGF- β 1/ β 2 precursor coding sequence controlled by expression regulatory elements. Simian TGF- β 1 cDNA (Sharples et al., 1987, DNA 6:239-244) was modified so that the nucleo- tides encoding amino acid residue numbers 9-13, 17, 19, 25 and 26 of the mature TGF- β 1 sequence were hanged to the nucleo- tides encoding the corresponding amino acids of the mature TGF- β 2 structure. Simian codon usage was maintained. The chimer- ic TGF- β 1/ β 2 of the invention induces effects on the proliferation of vascular endothelial cells equivalent to those induced by TGF- β 1 (see Section 7., <i>et seq.</i>). | | |

* (Referred to in PCT Gazette No. 29/1992, Section II)

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79:5312-5316; Assoian et al., 1983, J. Biol. Chem. 258:7155-7160) kidney (Roberts et al., 1983, Biochemistry 22:5692-5698), and demineralized bone (Seyedin et al., 1985, Proc. Natl. Acad. Sci. USA 82:119-123). cDNA clones coding for human (Derynck et al., 1985, Nature 316:701-705), mouse (Derynck et al., 1986, J. Biol. Chem. 261:4377-4379) and simian (Sharples et al., 1987, DNA 6:239-244) TGF- β 1 have been isolated. DNA sequence analysis of these clones indicates that TGF- β 1 is synthesized as a large precursor polypeptide, the carboxy terminus of which is cleaved to yield the mature TGF- β monomer. Strong sequence homology has been found throughout the TGF- β 1 precursor protein from all of the above sources.

15 In the presence of 10% serum and epidermal growth factor, TGF- β 1 promotes the anchorage independent growth of normal rat kidney fibroblasts (Roberts et al., 1981, Proc. Natl. Acad. Sci. USA 78:5339-5343; Roberts et al., 1982, Nature 295:417-419; Twardzik et al., 1985, J. Cell. Biochem. 28:289-297); in the presence of 10% serum alone, it is able to induce colony formation of AKR-2B fibroblasts (Tucker et al., 1983, Cancer Res. 43:1518-1586). TGF- β 1 has also been shown to cause fetal rat muscle mesenchymal cells to differentiate and produce cartilage specific macromolecules (Seyedin et al., 1986, J. Biol. Chem. 261:5693-5695).

In contrast to its effect on cell proliferation, TGF- β 1 purified from human platelets has been shown to inhibit the growth of certain cells in culture (Tucker et al., 1984, Science 226:705-707). TGF- β 1 has also been shown to inhibit the growth of several human cancer cell lines (Roberts et al., 1985, Proc. Natl. Acad. Sci. USA 82:119-123). This inhibitory/stimulatory effect of TGF- β 1 may depend on

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several factors including cell type and the physiological state of the cells (for review see Sporn et al., 1986, Science 233:532-534).

TGF- β 2, like TGF- β 1, is a polypeptide of
5 molecular weight 26,000 composed of two identical
13,000-dalton subunits which are disulfide linked
(Chieftetz et al., 1987, Cell 48:409-415; Ikeda et al.,
1987, Biochemistry 26:2406-2410) and has been isolated
from bovine demineralized bone (Seydin et al., 1987,
10 J. Biol. Chem. 262:1946-1949), porcine platelets
(Cheifetz et al., 1987, 48:409-415), a human prostatic
adenocarcinoma cell line, PC-3 (Ikeda et al., 1987,
Biochemistry 26:2406-2410), and a human glioblastoma
cell line (Wrann et al., 1987, EMBO 6:1633-1636).
15 cDNA clones coding for human and simian TGF- β 2 have
been isolated (Madisen et al., 1988, DNA 7:1-8; Webb
et al., 1988, DNA 7:493-497). The mature TGF- β 2
monomer is cleaved from one of two larger precursor
polypeptides, the mRNAs of which may arise via
20 differential splicing (Webb et al., 1988, DNA 7:493-
497).

TGF- β 1 and TGF- β 2 share 71% amino acid sequence
identity in their mature regions, and 41% identity in
their precursor structures. TGF- β 3, the amino acid
25 sequence of which has very recently been deduced from
cDNA clones, appears to contain a C-terminal 112 amino
acid sequence with about 80% homology to the mature
monomers of TGF- β 1 and TGF- β 2 (Dijke et al., 1988,
Proc. Natl. Acad. Sci. USA 85:4715-4719). TGF- β 1.2 is
30 a heterodimeric form comprising a β 1 and β 2 subunit
linked by disulfide bonds (Cheifetz et al., 1987, Cell
48:409-415).

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2.1 INTRACELLULAR PROCESSING OF TGF- β 1

The amino portion of the precursor region of TGF- β 1 from human, rodent and simian sources show a high
5 degree of homology (Derynck et al., 1985, Nature 316:701-705; Derynck et al., 1986, J. Biol. Chem. 261:4377-4379; Sharples et al., 1987, DNA 6:239-244), suggesting an important biological function may be associated with this part of the molecule. Recent
10 studies demonstrating that this portion of the TGF- β 1 precursor is glycosylated and phosphorylated support this contention since one might assume that a cell would not go through the expense of performing these secondary modifications were it not for a specific
15 function (Brunner et al., 1988, Mol. Cell. Biol. 8:2229-2232). These modifications may be important for dimerization of the precursor or for directing its movement out of the cell. There is evidence which suggests that glycosylation of the precursor is
20 involved in the transport of mature TGF- β 1 out of the cell (Purchio et al., 1988, J. Biol. Chem. 263:14211-14215).

The existence of what may either be intermediate precursor complexes involved in processing or
25 expression artifacts in CHO cells expressing the simian TGF- β 1 gene has been reported (Gentry et al., 1988, Mol. Cell. Biol. 8:4162-4168 press; Gentry et al., 1987, Mol. Cell. Biol. 7:3418-3427). These studies revealed that the TGF- β 1 precursor synthesized
30 by transfected CHO cells consists of pro-TGF- β 1, mature TGF- β 1, and the pro region of the precursor interlinked by disulfide bonds. Such disulfide-linked precursor complexes have also been observed in isolated latent forms of TGF- β 1 (Miyazano et al.,
35

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1988, J. Cell. Biochem. Suppl. 12(A):200; Wakefield et al., 1987, J. Biol. Chem. Suppl. 11(A):46).

Gentry et al. (Gentry et al., 1988, Mol. Cell. Biol., 8:4162-4168) have proposed the following scheme
5 for the processing of pre-pro-TGF- β 1 in transfected CHO cells. (The amino acid position numbers referred to are from the published sequence of simian TGF- β 1 (Sharples et al., 1987, DNA 6:239-244)). According to
10 this proposed scheme, the first step involves signal peptide cleavage at the Gly-29/Leu-30 peptide bond. This cleavage event most likely occurs co-translationally during transit of the precursor through the rough endoplasmic reticulum membrane (Blobel and Dobberstein, 1975, J. Cell. Biol. 67:835-
15 851; Walter et al., 1984, Cell 38:5-8). Following cleavage of the signal peptide, core glycosylation units (Rothman et al., 1978, Cell 15:1447-1454) are added to pro-TGF- β 1 at each of three predicted N-glycosylation sites located at Asn-82, Asn-136 and
20 Asn-176. The core glycosylated pro-TGF- β 1 is then sequentially processed during transit through the Golgi to yield a phosphorylated glycoprotein containing complex, sialated oligosaccharides. At some stage during synthesis or transit, proteolytic
25 cleavage at the dibasic residue and disulfide isomerization occurs, releasing mature TGF- β 1.

In another recent study, mannose-6-phosphate was identified in the TGF- β 1 precursor. Mannose-6-phosphate, a phosphorylated sugar analog, appears to
30 play a fundamental role in the targeted transport and intercellular exchange of lysosomal enzymes (von Figura, 1986, Ann. Rev. Biochem. 55: 167-193). Specific receptors which recognize the mannose-6-phosphate residues of lysosomal enzymes have been
35 identified and are essential components of the

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transport system. Secreted lysosomal proteins containing mannose-6-phosphate have been identified in the conditioned medium of tissue culture cells (Gal and Gottesman, 1986, J. Biol. Chem. 261:1760-1765; 5 Capony et al., 1981, J. Cell. Biol. 104:253-262; Baumbach et al., 1984, Proc. Natl. Acad. Sci. USA 81:2985-2989; Sahagian and Gottesman, 1982, J. Biol. Chem. 257:11145-11150). It is possible that the mannose-6-phosphate residues of the TGF- β 1 precursor 10 may direct pro-TGF- β 1 to lysosomes for proteolytic processing to yield mature TGF- β 1. Alternatively, the mannose-6-phosphate residues may function to target the cleaved TGF- β 1 precursor to lysosomes for degradation.

15

3. SUMMARY OF THE INVENTION

The present invention relates to the production of large quantities of a novel chimeric TGF- β , termed TGF- β 1/ β 2, by eucaryotic host cells transfected with 20 recombinant DNA vectors containing the TGF- β 1/ β 2 precursor coding sequence controlled by expression regulatory elements. Simian TGF- β 1 cDNA (Sharples et al., 1987, DNA 6:239-244) was modified so that the nucleotides encoding amino acid residue numbers 9-13, 25 17, 19, 25 and 26 of the mature TGF- β 1 sequence were changed to the nucleotides encoding the corresponding amino acids of the mature TGF- β 2 structure. Simian codon usage was maintained.

Expression vectors encoding the chimeric TGF- 30 β 1/ β 2 precursor under the regulatory control of Simian Virus 40 (SV 40) expression regulatory elements were constructed and used to transfect Chinese Hamster ovary (CHO) cells. CHO transfectants which synthesize and secrete high levels of mature TGF- β 1/ β 2 were 35 obtained. TGF- β 1/ β 2 expression was amplified with

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methotrexate and amplified transfectants secreted as much as 1mg/L mature TGF- β 1/ β 2. Acidification of the conditioned media of the CHO transfectants resulted in maximal levels of bioactive TGF- β 1/ β 2. It is believed
5 that the high levels of mature TGF- β 1/ β 2 secreted by the transfected CHO cells results from an unusual efficiency in the proteolytic processing of the chimeric precursor protein. Such increased processing efficiency may, in turn, result from structural
10 characteristics affected by applicants' combination of the TGF- β 1 and TGF- β 2 amino acid sequences in the amino-terminal domain of the mature TGF- β structure.

The chimeric TGF- β 1/ β 2 of the invention induces effects on the proliferation of vascular endothelial
15 cells equivalent to those induced by TGF- β 1 (see Section 7., et seq.).

4. DESCRIPTION OF THE FIGURES

FIG. 1. Nucleotide and deduced amino acid
20 sequence of the TGF- β 1/ β 2 hybrid protein encoded by expression plasmid p5 β /dhfr.

FIG. 2. Bioactivity of conditioned media from 5 β 41,2.5 cells. Bioactivity was measured by the growth inhibition assay of CCL-64 mink lung epithelial
25 cells. (A) Serum-free media conditioned by 5 β 41,2.5 cells for 24 hours was dialyzed against 0.2 M acetic acid and assayed as described in Section 6.1.3., infra. (B) Standard growth inhibition curve for TGF- β 1.

30 FIG. 3. Immunoblot analysis of proteins secreted by 5 β 41,2.5 cells. 5 β 41,2.5 cells were grown to confluence; media was dialyzed against 0.2 M acetic acid and assayed by immunoblotting under nonreducing (lane 1) or reducing (lane 2) conditions with anti-
35 TGF- β 1₃₆₉₋₃₈₁ as described in Section 6.1.5., infra.

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FIG. 4. Effect of various TGF- β s on vascular endothelial cells. Two-dimensional, five-day vascular cell cultures were grown in DME plus 10% FCS (control) with additions of TGF- β 1, TGF- β 2, both at 0.5 ng/ml, or TGF-5 β at concentrations of 0.05, 0.5 or 5.0 ng/ml. (A) BAEC; (B) BASMC; (C) RFC. In all cases the TGF-5 β hybrid molecule mimicked the TGF- β 1 isoform.

FIG. 5. Effect of various TGF- β s on vascular cell migration. Migrating aortic endothelial and smooth muscle cells were treated with TGF- β 1 and TGF- β 2 at 0.5 ng/ml and TGF-5 β at concentrations of 0.05, 0.5 or 5.0 ng/ml. TGF-5 β mimicked the TGF- β 1 response induced on BASMCs by increasing migratory rates, while the opposite effect was seen when using large vessel endothelial cells. TGF- β 2 elicited no effect on either cell type.

FIG. 6. Angiogenic response induced by various TGF- β s. Three-dimensional RFC cultures were viewed with Hoffman interference microscopy to analyze angiogenic responses elicited by TGF- β 1, TGF- β 2, and TGF-5 β . Four-day, 3-D small vessel endothelial cultures were cryosectioned at 8 μ m and acetone fixed. Control cultures (A) were grown in DME plus 10% FCS. Treated cultures included: (B) TGF- β 1 (0.5 ng/ml); (C) TGF- β 2 (0.5 ng/ml); and TGF-5 β at (D) 0.05 ng/ml; (E) 0.5 ng/ml and (F) 5.0 ng/ml. TGF- β 1 evokes complex, branching tubular structures, while the control cultures showed minimal tube formation. TGF-5 β at 0.05 ng/ml gave an indication of commencing neovascularization, while 0.5 ng/ml of the hybrid molecule stimulated an angiogenic response equivalent to the TGF- β 1 isoform. The highest concentration of TGF-5 β appears to cause a slight decrease in the amount of complex branching structures which had been shown before using TGF- β 1. TGF- β 2 required a 10-fold

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increase in concentration (5.0 ng/ml) in order to match the neovascularization induced by TGF- β 1 and TGF- β 2 at 0.5 ng/ml. Arrows indicate tubular formations by RFCs. Magnification = 100 X.

5

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to TGF- β 1/ β 2, to nucleotide sequences encoding TGF- β 1/ β 2 and the TGF- β 1/ β 2 precursor, and to the production of TGF- β 1/ β 2 by recombinant DNA methods. TGF- β 1/ β 2, a novel chimeric transforming growth factor-beta, is biologically active in the standard assay used to measure TGF- β 1 bioactivity and is immunoreactive with TGF- β 1-specific antibodies. A chimera structurally comprising a combination of TGF- β 1 and TGF- β 2 amino acid sequences, the TGF- β 1/ β 2 of the invention is likely to carry a novel portfolio of biological activities, some of which may be similar or nearly identical to those exhibited by its parent molecules while others may be unique to TGF- β 1/ β 2. With regard to those bioactivities which are similar or nearly identical to those of TGF- β 1 or TGF- β 2, this new factor may provide a more effective means of inducing corresponding biological responses and its use may therefore be a desirable alternative to TGF- β 1 and TGF- β 2 in various medical applications envisioned for the TGF- β s. Such applications include but are not limited to inducing or accelerating cell proliferation and differentiation and, inhibiting cell division. Thus TGF- β 1/ β 2 may find uses in, for example, treating cancer and promoting wound healing.

The method of the invention may be divided into the following stages solely for the purposes of description: (a) generation of the coding sequence for the TGF- β 1/ β 2 precursor; (b) construction of an

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expression vector which will direct the expression of the TGF- β 1/ β 2 coding sequence; (c) transfection of appropriate host cells which are capable of replicating, expressing the gene and processing the gene product to produce the mature form of TGF- β 1/ β 2 and/or TGF- β 1/ β 2 precursors; and (d) identification and purification of the TGF- β 1/ β 2 precursors and the mature, biologically active TGF- β 1/ β 2.

Once a transfectant is identified that expresses high levels of TGF- β 1/ β 2 precursors and/or mature TGF- β 1/ β 2, the practice of the method of the invention involves the expansion of that clone and isolation of the gene product expressed.

The method of the invention is demonstrated herein, by way of examples in which simian TGF- β 1 precursor cDNA (Sharples et al., 1987, DNA 6:239-244) is modified so that the nucleotides encoding amino acid residue numbers 9-13, 17, 19, 25 and 26 of the mature simian TGF- β 1 sequence are changed to the nucleotides encoding the corresponding amino acids in the mature TGF- β 2 structure, while maintaining simian codon usage. The resulting chimeric TGF- β 1/ β 2 precursor coding sequence is then used to construct expression vectors which are capable of directing the synthesis of the mature TGF- β 1/ β 2 product.

The effects of TGF- β 1/ β 2 on the proliferation, migration and angiogenesis of vascular endothelial cells have been examined in vitro (section 7., et seq.) and appear to closely parallel those induced by TGF- β 1 but not TGF- β 2.

The various aspects of the method of the invention are described in more detail in the subsections below and in the examples that follow.

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5.1 GENERATION OF THE CHIMERIC TGF- β 1/ β 2 CODING SEQUENCE

The nucleotide coding sequence for the chimeric
5 TGF- β 1/ β 2 is depicted in FIG. 1. In the practice of
the method of the invention, this nucleotide sequence
or its functional equivalent can be used to generate
the recombinant molecules which will direct the
expression of the TGF- β 1/ β 2 product. Due to the
10 degeneracy of the nucleotide coding sequences, other
DNA sequences as depicted in FIG. 1 may be used in the
practice of the present invention. Such alterations
of the nucleotide sequence of FIG. 1 include
deletions, additions or substitutions of different
15 nucleotide residues resulting in a sequence that
encodes the same or a functionally equivalent gene
product. The gene product may contain deletions,
additions or substitutions of amino acid residues
within a sequence, which result in a silent change
20 thus producing a bioactive product. Such amino acid
substitutions may be made on the basis of similarity
in polarity, charge, solubility, hydrophobicity,
hydrophobicity and/or the amphipathic nature of the
residues involved. For example, negatively charged
25 amino acids include aspartic acid and glutamic acid;
positively charged amino acids include lysine and
arginine; amino acids with uncharge dipolar head
groups or nonpolar head groups having similar
hydrophilicity values include the following: leucine,
30 isoleucine, valine; glycine, alanine; asparagine,
glutamine; serine, threonine; phenylalanine, tyrosine.

The nucleotide sequence for simian TGF- β 1 may be
obtained from simian cell sources (Sharples et al.,
1989, DNA 6:239-244). The nucleotide sequence of the
35 chimeric TGF- β 1/ β 2 in FIG. 1 may be prepared by
methods known in the art including but not limited to

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the use of DNA restriction enzymes, synthetic oligonucleotides, and DNA ligases. Alternatively, the coding sequence of FIG. 1 may be synthesized in whole or in part using chemical methods well known in the art.

In a specific embodiment of the invention, the coding sequence for simian TGF- β 1 was obtained from a full length cDNA clone obtained from an African green monkey cell line, BSC-40 (Sharples et al., 1987, supra). The coding sequence of chimeric TGF- β 1/ β 2 depicted in FIG. 1 was then derived from the simian TGF- β 1 cDNA by removing and replacing the coding sequences of amino acid residue numbers 9, 10, 11, 12, 13, 17, 19, 25 and 26 of the mature TGF- β 1 molecule with the coding sequences for amino acid residue numbers 9, 10, 11, 12, 13, 17, 19, 25 and 26 of the mature TGF- β 2 molecule (Madisen et al., 1988, DNA 7:1-8) using gene construction techniques.

5.2 CONSTRUCTION OF EXPRESSION VECTORS CONTAINING THE CHIMERIC TGF- β 1/ β 2 CODING SEQUENCE

In order to express biologically active, mature TGF- β 1/ β 2, an expression vector/host system should be chosen which provides not only for high levels of transcription and translation but for the correct processing of the gene product. This is especially important when employing the entire coding sequence of the chimeric TGF- β 1/ β 2 precursor in the expression constructs because, like TGF- β 1 and TGF- β 2, the mature chimeric TGF- β 1/ β 2 is believed to be released from a precursor molecule or complex of molecules via cellular processing events. In addition, an expression/host cell system which provides for secretion of the product may be desirable.

In particular, it appears that mature TGF- β 1/ β 2 is a disulfide linked homodimer of 112 amino acids per

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subunit formed by cellular processing events believed to be similar to those which form mature TGF- β 1 and TGF- β 2. The TGF- β 1/ β 2 precursor has three potential N-glycosylation sites in its pro domain (Sharples et al., 1987, DNA 6:239-244). Studies involving TGF- β 1 have determined that N-glycosylation and phosphorylation in the pro domain of TGF- β 1 occurs in transfected CHO cells, implicating an important functional role for the precursor in the cellular synthesis and release or secretion of the mature molecule (Brunner et al., 1988, Mol. Cell. Biol. 8:2229-2232). The presence of mannose-6-phosphate in the TGF- β 1 precursor also supports the hypothesis that the precursor has independent functional activity (Purchio et al., 1988, J. Biol. Chem. 263:14211-14215). Since the chimeric TGF- β 1/ β 2 precursor contains the simian TGF- β 1 pro domain, applicants believe it likely that the TGF- β 1/ β 2 precursor is functionally active and important to the correct processing the mature TGF- β 1/ β 2 molecule. Thus, the ability of a host cell used in the expression system to correctly express and process chimeric TGF- β 1/ β 2 is important to the production of a mature, bioactive product.

In a specific embodiment described herein, mature bioactive TGF- β 1/ β 2 is successfully produced using simian virus 40 (SV40) expression control elements in a Chinese Hamster Ovary (CHO) host cell system. However, a variety of other animal host/expression vector systems (i.e., vectors which contain the necessary elements for directing the replication, transcription and translation of the TGF- β 1/ β 2 coding sequence in an appropriate host cell) may be utilized equally well by the skilled artisan. These include, but are not limited to, virus expression

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vector/mammalian host cell systems (e.g., cytomegalovirus, vaccinia virus, adenovirus, and the like); insect virus expression vector/insect cell systems (e.g., baculovirus); or nonviral promoter
5 expression systems derived from the genomes of mammalian cells (e.g., the mouse metallothionein promoter).

The expression elements of these vectors vary in their strength and specificities. Depending on the
10 host/vector system utilized, any one of a number of suitable transcription and translation elements may be used. For instance, when cloning in mammalian cell systems, promoters isolated from the genome of mammalian cells, (e.g. mouse metallothionein promoter)
15 or from viruses that grow in these cells, (e.g. vaccinia virus 7.5K promoter) may be used. Promoters produced by recombinant DNA or synthetic techniques may also be used to provide for transcription of the inserted sequences.

20 Specific initiation signals are also required for sufficient translation of inserted protein coding sequences. These signals include the ATG initiation codon and adjacent sequences. For example, in cases where only a portion of the TGF- β 1/ β 2 coding sequence
25 is inserted, exogenous translational control signals, including the ATG initiation codon must be provided. Furthermore, the initiation codon must be in phase with the reading frame of the TGF- β 1/ β 2 coding sequences to ensure translation of the entire insert.
30 These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of transcription attenuation sequences, enhancer elements, and the
35 like.

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Any of the methods previously described for the insertion of DNA fragments into a vector may be used to construct expression vectors containing the TGF- β 1/ β 2 coding sequence and appropriate

5 transcriptional/translational control signals. These methods may include in vitro recombinant DNA techniques, synthetic techniques and in vivo recombinations (genetic recombination).

In cases where an adenovirus is used as an
10 expression vector, the TGF- β 1/ β 2 coding sequence may be ligated to an adenovirus transcription/translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by in vitro
15 or in vivo recombination. Insertion in a non-essential region of the viral genome (e.g., region E1 or E3) will result in a recombinant virus that is viable and capable of expressing chimeric TGF- β 1/ β 2 in infected hosts. Similarly, the vaccinia 7.5K promoter
20 may be used.

An alternative expression system which could be used to express TGF- β 1/ β 2 is an insect system. In one such system, Autographa californica nuclear polyhydrosis virus (AcNPV) is used as a vector to
25 express foreign genes. The virus grows in Spodoptera frugiperda cells. The TGF- β 1/ β 2 coding sequence may be cloned into non-essential regions (for example, the polyhedrin gene) of the virus and placed under the control of an AcNPV promoter (for example, the
30 polyhedrin promoter). Successful insertion of the TGF- β 1/ β 2 coding sequence will result in inactivation of the polyhedrin gene and production of non-occluded recombinant virus (i.e., virus lacking the proteinaceous coat encoded by the polyhedrin gene).
35 These recombinant viruses are then used to infect

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Spodoptera frugiperda cells in which the inserted gene is expressed.

In addition, a host cell strain may be chosen which modulates the expression of the inserted
5 sequences, or modifies and processes the gene product in the specific fashion desired. Expression from certain promoters can be elevated in the presence of certain inducers, (e.g. zinc and cadmium ions for metallothionein promoters). Therefore, expression of
10 the genetically engineered TGF- β 1/ β 2 may be controlled. This is important if the protein product of the cloned foreign gene is lethal to host cells. Furthermore, post-translational modifications such as glycosylation, and processing events such as
15 proteolytic cleavage of protein products, may be important to the functionality of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins. Appropriate cell lines or
20 host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed.

In a specific embodiment of the invention, an expression vector containing the TGF- β 1/ β 2 coding
25 sequence in tandem with the mouse dihydrofolate reductase gene (dhfr) under the control of SV40 regulatory sequences is constructed and used to transfect dhfr-deficient CHO cells. CHO transfectants expressing the dhfr phenotype are isolated by
30 propagation in selective media. To increase the level of expression of TGF- β 1/ β 2, transfectants may be exposed to increasing concentrations of methotrexate in order to isolate clones transcribing amplified levels of TGF- β 1/ β 2 mRNA. TGF- β 1/ β 2 mRNA levels may
35 be assayed at various stages of amplification by

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solution hybridization (Uhler et al., 1986, Proc. Natl. Acad. Sci. U.S.A. 83:1300-1304).

5.3 IDENTIFICATION OF TRANSFECTANTS EXPRESSING CHIMERIC TGF- β 1/ β 2

5 The host cells which contain the TGF- β 1/ β 2 coding sequence and which express the biologically active, mature product may be identified by at least four general approaches: (a) DNA-DNA hybridization; (b) the
10 presence or absence of "marker" gene functions; (c) assessing the level of transcription as measured by the expression of TGF- β 1/ β 2 mRNA transcripts in the host cell; and (d) detection of the mature gene product as measured by immunoassay and, ultimately, by
15 its biological activities.

In the first approach, the presence of the TGF- β 1/ β 2 coding sequence inserted in the expression vector can be detected by DNA-DNA hybridization using probes comprising nucleotide sequences that are
20 homologous to the TGF- β 1/ β 2 coding sequence substantially as shown in FIG. 1, or portions or derivatives thereof.

In the second approach, the recombinant expression vector/host system can be identified and
25 selected based upon the presence or absence of certain "marker" gene functions (e.g., thymidine kinase activity, resistance to antibiotics, resistance to methotrexate, transformation phenotype, occlusion body formation in baculovirus, etc.). For example, if the
30 TGF- β 1/ β 2 coding sequence is inserted within a marker gene sequence of the vector, recombinants containing the TGF- β 1/ β 2 coding sequence can be identified by the absence of the marker gene function. Alternatively, a marker gene can be placed in tandem with the TGF- β 1/ β 2
35 sequence under the control of the same or different promoter used to control the expression of the TGF-

$\beta 1/\beta 2$ coding sequence. Expression of the marker in response to induction or selection indicates expression of the TGF- $\beta 1/\beta 2$ coding sequence.

In the third approach, transcriptional activity
5 for the TGF- $\beta 1/\beta 2$ coding region can be assessed by hybridization assays. For example, polyadenylated RNA can be isolated and analyzed by Northern blot using a probe homologous to the TGF- $\beta 1/\beta 2$ coding sequence or particular portions thereof. Alternatively, total
10 nucleic acids of the host cell may be extracted and assayed for hybridization to such probes.

In the fourth approach, the expression of the mature protein product can be assessed immunologically, for example by Western blots,
15 immunoassays such as immunoblotting, radioimmunoprecipitation, enzyme-linked immunoassays, and the like. The ultimate test of the success of the expression system, however, involves the detection of the biologically active TGF- $\beta 1/\beta 2$ gene product. Where
20 the host cell secretes the gene product, the cell free media obtained from the cultured transfectant host cell may be assayed for TGF- $\beta 1/\beta 2$ activity. Where the gene product is not secreted, cell lysates may be assayed for such activity. In either case, biological
25 assays such as the growth inhibition assay described herein or the like may be used.

Once a clone producing high levels of mature TGF- $\beta 1/\beta 2$ is identified, the clone may be expanded and the TGF- $\beta 1/\beta 2$ may be purified using techniques well known
30 in the art. Such methods include immunoaffinity purification, chromatographic methods including high performance liquid chromatography, and the like.

5.4 IMPROVED METHOD FOR PRODUCING MATURE TGF- β 1/ β 2

In a specific embodiment of the invention, the TGF- β 1/ β 2 precursor may be modified by eliminating the amino acid cysteine at position 33 (CYS-33) of FIG. 1 and replacing it with serine. This modification may be introduced at the DNA level using site directed mutagenesis. COS cells transfected with plasmids encoding the TGF- β 1 precursor gene so modified ultimately secrete between three and five times more mature TGF- β 1 than do CHO cells expressing the unmodified precursor gene. It is believed that this modification prevents the formation of certain disulfide-linked precursor complexes in the transfected COS cells and therefore allows for the secretion of more mature TGF- β .

6. EXAMPLE: PRODUCTION OF TGF- β 1/ β 2 BY EXPRESSION IN CHINESE HAMSTER OVARY CELLS

A recombinant plasmid encoding TGF- β 1 precursor in which amino acids 9, 10, 11, 12, 13, 17, 19, 25 and 26 of the mature TGF- β 1 sequence were replaced by the corresponding amino acids of the mature TGF- β 2 sequence was constructed. Specifically, amino acid 9 of mature TGF- β 1 (serine) was replaced by arginine, amino acid number 10 (serine) was replaced by asparagine, amino acid number 11 (threonine) was replaced by valine, amino acid number 12 (glutamic acid) was replaced by glutamine, amino acid number 13 (lysine) was replaced by aspartic acid, amino acid number 17 (valine) was replaced by leucine, amino acid number 19 (glutamine) was replaced by proline, amino acid number 25 (arginine) was replaced by lysine and amino acid number 26 (lysine) was replaced by arginine. The construct was used to transfect CHO

cells. Transfectants which produced and secreted a mature, bioactive, chimeric TGF- β 1/ β 2 were isolated.

6.1 MATERIALS AND METHODS

5

6.1.1 DNA TRANSFECTIONS

Approximately 24 hours after seeding 10^6 dhfr-deficient CHO cells onto 100 mm dishes, the cultures were transfected with 1 μ g of NdeI linearized p5 β /dhfr
10 plasmid and 19 μ g of calf thymus DNA as carrier as a calcium phosphate precipitate (Wigler, M., et al., 1979, Proc. Natl. Acad. Sci. U.S.A. 76:1373-1376). Briefly, 20 μ g of plasmid plus carrier DNA was added to 1 ml of 250 mM sterile CaCl₂. The DNA solution (1
15 ml) was added dropwise to a 1 ml portion of 2X HEPES solution (280 mM NaCl, 50 mM HEPES, 1.5 mM sodium phosphate, pH 7.1) while bubbling and the mixture was allowed to sit on ice for 30 minutes. The precipitate was then dispersed dropwise over the cells containing
20 10 ml of F12 media (Gibco). After incubation at 37°C for 4 hours, the media was removed and replaced with 10 ml of F12 media containing 25% glycerol for 90 seconds at room temperature. Cells were rinsed once with 20 ml of F12 media and incubated in the
25 nonselective F12 media (20 ml) for an additional 48 hours. Selection for dhfr expressing transfectants was accomplished by replacing the media with DMEM supplemented with 10% dialyzed FBS (Gibco) and 150 μ g/ml L-proline. Colonies were observed after
30 culturing the cells 10-14 days in the selection media.

6.1.2 SELECTION OF METHOTREXATE RESISTANT CELLS

Dihydrofolate reductase (dhfr) amplified cells were derived from the primary transfectants
35 essentially as described (Gasser, C.S. and Schimke,

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R.T., 1986, J. Biol. Chem. 261:6938-6946). After expansion, 10^5 cells were seeded onto 100 mm dishes and adapted to increasing concentrations of methotrexate (100 nM; 500 nM; 2,500 nM; 10,000 nM; 20,000 nM). The
5 initial concentration of methotrexate was 100 nM. The plate containing visible colonies was trypsinized and adapted to that concentration of methotrexate for at least two additional 1:5 cell passages. Cells (10^5) were then seeded onto 100 mm dishes in the next
10 highest concentration of methotrexate. The dish containing visible colonies was again trypsinized and adapted in the methotrexate containing medium. Cells were frozen back at various stages of amplification in media containing 40% FBS, 10% dimethyl sulfoxide and
15 50% DMEM. Methotrexate was not included in the freezing media.

6.1.3 GROWTH INHIBITION ASSAY

Mink lung epithelial cells, Mv 1 Lu (Accession
20 Number CCL-64, American Type Culture Collection), which are extremely sensitive to TGF- β were utilized for the growth inhibition assay. The assay was performed using the thymidine analog 5'-[125 I]-iodo-2'deoxyuridine (125 IdU) to assess DNA synthesis. One
25 unit of activity was defined as the amount required to inhibit 50% incorporation of 125 IdU compared to untreated CCL-64 cells.

To assay transfected cells for secretion of active TGF- β 1/ β 2, serum free supernatants were
30 collected from one 24 hour collection on confluent cultures of cells and dialyzed extensively against 0.2 M acetic acid. Samples were diluted into sterile complete culture medium for assays.

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6.1.4 PEPTIDE SYNTHESIS AND PRODUCTION OF ANTIBODIES

Peptides were synthesized by solid phase techniques on a Beckman 990 instrument, and cleaved
5 from the resin as previously described (Gentry, L.E., et al., 1983, J. Biol. Chem. 258:11219-11228; Gentry, L.E. and Lawton, A., 1986, Virology 152:421-431). Purification was accomplished by preparative high performance liquid chromatography. The composition of
10 the peptides was confirmed by amino acid analysis.

Synthetic peptides were conjugated to bovine gamma-globulin through the cysteine residue. Coupling reactions were performed essentially as described (Gentry and Lawton, 1986, supra). The efficiencies of
15 peptide conjugations ranged from 8 to 26 molecules of peptide covalently attached per molecule of gamma-globulin.

New Zealand white rabbits were primed at three to six sites by combined subcutaneous and intradermal
20 inoculations with the peptide conjugates (100 ug equivalents of peptide) emulsified in Freund's complete adjuvant. Booster inoculations were administered at 2-3 week intervals. Bleedings were taken 7-14 days following the boosts.

25 Anti-peptide antibodies directed toward peptide sequences within the TGF- β 1 molecule were generated in rabbits using synthetic peptides as immunogens (Gentry et al., 1987, Mol. Cell. Biol. 7:3418-3427). One of the antibodies (anti-TGF- β 1₃₆₉₋₃₈₁) was directed toward
30 epitopes present within the mature form of the TGF- β growth factor. The other two antibodies (anti-TGF- β 1₈₁₋₉₄ and anti-TGF- β 1₂₂₅₋₂₃₆) are precursor-specific and are directed toward peptide sequences present only within the precursor molecule of TGF- β 1.

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6.1.5 IMMUNOBLOTTING

Proteins were fractionated on 7.5%-17.5% gradient SDS-polyacrylamide gels and transferred to unmodified nitrocellulose (0.45 μ m; Schleicher and Schuell) for 1 hour at 24 volts at 4°C (Burnette, W.N., 1981, Anal. Biochem. 112:195-203). Excess binding capacity of the nitrocellulose was blocked by incubation with 2.5% BLOTTO (Johnson, D.A., et al., 1984, Gene Anal. Techn. 1:3-8) in phosphate-buffered saline (PBS) containing 0.2% NP-40. Rabbit anti-serum diluted 1:75 in 2.5% BLOTTO was incubated with the blocked nitrocellulose sheets for 2 hours at room temperature. After washing away excess antibody by five 5-minute washes in 2.5% BLOTTO, the nitrocellulose sheets were incubated with alkaline phosphatase-conjugated Protein A diluted 1:500 in 2.5% BLOTTO. Following a two hour incubation, the nitrocellulose sheets were washed 5 times in PBS (5 minute washes) containing 0.2% NP-40 and developed (Leary et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:4045-4049).

6.1.6 CONSTRUCTION OF PLASMID PROGRAMMING THE SYNTHESIS OF TGF- β 1/ β 2

The plasmid programming the synthesis of the chimeric TGF- β 1/ β 2 protein, p5 β /dhfr, was constructed as follows. pAc β TGF-1, a baculovirus vector derived from pAc373 (Miyamoto et al., 1985, Cell. Biol. 5:2860-2865; Madisen et al., 1987, Virology 158:248-250), which contains the 1.4 Kb PstI-EcoRI coding sequence of TGF- β 1 (Sharples et al., 1987, DNA 6:239-244) cloned into the PstI-EcoRI site of pAc611 (Miyamoto et al., 1985, Cell. Biol. 5:2860-2865; Madisen et al., 1987, Virology 158:248-250), was digested with BamHI and EcoRI and the 375 bp fragment of the TGF- β 1 coding sequence was isolated (Fragment 1). pSV2- β TGF (Gentry et al., 1987, Mol. Cell. Biol.

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7:3418-3427) was digested with ApaI and EcoRI and the 3.5 Kb fragment was isolated (Fragment 2).

Complementary synthetic oligonucleotides having the sequences shown below were synthesized on an
 5 Applied Biosystems Oligonucleotide Synthesizer and purified from an acrylamide gel. Phosphates were added with T4 kinase and equimolar amounts of the kinased oligonucleotides were annealed. The annealed double stranded synthetic DNA was then ligated to
 10 fragments '1' and '2' described above. The ligation mixture was used to transform E. coli and 5 β pSV2(Hpa⁻Eco⁺) was isolated.

15 5' - CAA CAT CTG CAA AGC TCC CGG CAC CGC CGA GCC
CTG GAC ACC AAC TAC TGC TTC AGA AAT GTG CAG
GAT AAT TGC TGC CTA CGT CCG CTT TAC ATT GAT
TTC AAG AGG GAT CTA GGG TGG AAA TG - 3'

20 5' GAT CCA TTT CCA CCC TAG ATC CCT CTT GAA ATC
AAT GTA AAG CGG ACG TAG GCA GCA ATT ATC CTG
CAC ATT TCT GAA GCA GTA GTT GGT GTC CAG GGC
TCG GCG GTG CCG GGA GCT TTG CAG ATG TTG GGC C - 3'

25 5 β pSV2(Hpa⁻Eco⁺) was digested with EcoRI, filled in with Klenow enzyme, digested with HindIII and the 1.4 Kb fragment containing the chimeric TGF- β 1/ β 2 coding sequence was isolated (Fragment 3). 5 β pSV2 was constructed by ligating Fragment 3 into pSV2,neo which had previously been digested with HindIII and HpaI to
 30 eliminate the neo gene.

5 β pSV2 was digested with EcoRI, filled in with Klenow enzyme, digested with NdeI and the 2.6 Kb NdeI-EcoRI (blunt) fragment was isolated and ligated to pSV2/dhfr (Gentry et al., 1987, Mol. Cell. Biol.
 35 7:3718-3727) which had been digested with NdeI and

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PvuII. The ligation mixture was used to transform E. coli and p5 β /dhfr was isolated. The nucleotide and deduced amino acid sequences of the chimeric TGF- β 1/ β 2 molecule encoded by p5 β /dhfr are shown in FIG. 1.

5

6.2 EXPRESSION OF TGF- β 1/ β 2 IN CHO CELLS

p5 β /dhfr was transfected into CHO cells and single clones were amplified with methotrexate as described in Section 6.1., supra. One such amplified
10 clone, CHO-5 β 41,2.5, was chosen for further characterization.

CHO-5 β 41,2.5 cells were grown to confluence in 2.5 μ M methotrexate. Media was replaced with serum free media and, after 24 hr, was collected and
15 dialyzed for 48 hr against 0.2M acetic acid. Dialyzed, conditioned supernatants were assayed for bioactivity by inhibition of DNA synthesis of CCL-64 cells as described in Section 6.1.3., supra. CHO-5 β 41,2.5 cells secrete approximately 1 mg/L of
20 bioactive chimeric TGF- β 1/ β 2 (FIG. 2).

TGF- β related proteins secreted by these cells were analyzed by immunoblotting using anti-peptide antibodies directed against mature TGF- β 1 as described in Section 6.1.5., supra. FIG. 3 shows that CHO-
25 5 β 41,2.5 cells secrete immunoreactive proteins migrating at 90 to 100 kilodaltons and at 24 kilodaltons when analyzed on SDS-PAGE under nonreducing conditions (FIG. 3, lane 1). The 24 kilodalton band represents the mature TGF- β 1/ β 2 dimer
30 and the 90 to 100 kilodalton protein probably represents mature TGF- β 1/ β 2 disulfide-bonded to precursor sequences (Gentry et al., 1987, Mol. Cell. Biol. 7:3418-3427).

Under reducing conditions (FIG. 3, lane 2), the
35 majority of the proteins migrate at 12 kilodaltons,

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representing the mature TGF- β 1/ β 2 monomer. Note the lack of immunoreactive material in the 45 to 55 kilodalton range observed in a similar analysis of recombinant proteins expressed in CHO cells transfected with plasmids encoding the simian TGF- β 1 gene (Gentry et al., 1987, Mol. Cell. Biol. 7:3418-3427) suggesting that chimeric TGF- β 1/ β 2 is proteolytically processed more efficiently than its parent molecule TGF- β 1. In addition, CHO-5 β 41,2.5 cells secrete about 2.5 times more bioactive mature product than do CHO cells expressing TGF- β 1 (Gentry et al., 1987, supra). Although the basis for these observations is presently unknown, the secondary structure of the chimeric TGF- β 1/ β 2 precursor may significantly differ from the secondary structure of TGF- β 1, which secondary structure renders the chimeric TGF- β 1/ β 2 subject to molecular processing events of a different intensity or nature. For example, the TGF- β 1/ β 2 precursor may be a more favorable substrate for the factors involved in TGF- β processing. Alternatively, the secondary structural characteristics of TGF- β 1/ β 2 may allow it to interact with other processing factors or pathways not as accessible to TGF- β 1.

25

7. EXAMPLE: GROWTH MODULATION OF VASCULAR
ENDOTHELIAL CELLS BY HYBRID TGF-5 β

Using three distinct bioassays which previously indicated that differential responses are induced by TGF- β 1 and TGF- β 2 (Merwin et al., 1991, Am. J. Path. 138(1):37-51), the biological effects induced by recombinant hybrid TGF- β 1/ β 2 (TGF-5 β) on the proliferation, migration and angiogenesis of large vessel endothelial, smooth muscle and microvascular endothelial cells were examined.

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7.1 MATERIALS AND METHODS

7.1.1 CELL CULTURE

Bovine aortic smooth muscle cells (BASMCs) were
5 grown from explants of bovine aorta in complete DME
supplemented with 10% FCS as described in Kocher and
Madri, 1989, In Vitro Cell. & Dev. Biol. 25:424-34.
After several days in culture, BASMCs migrate out from
the medial explants. Following the development of
10 confluency, the cells were trypsinized, passaged and
utilized in the growth inhibitory assays described,
infra. These α -smooth muscle, actin positive cells
formed the hill-and-valley pattern typical of cultured
smooth muscle cells and were used between passage 2
15 and 5.

Capillary endothelial cells were isolated and
cultured from rat epididymal fat pads as described in
Madri and Williams, 1983, J. Cell Biol. 97:153-65, and
are hereinafter termed "RFCs". Bovine calf aortic
20 endothelial cells (BAECs) were isolated and cultured
as described in Madri and Furthmayr, 1980, Hum. Path.
11:353-66. Three-dimensional RFC cultures were
composed of acid soluble calf dermis collagen type I
and prepared using the method of Madri et al., 1988,
25 J. Cell Biol. 106:1375-84. To address the possibility
of differences due to the use of two species,
proliferative, migratory and angiogenic studies were
also performed using bovine adrenal cortex
microvascular endothelial cells; the results were
30 equivalent to those obtained with RFCs.

7.1.2 TGF- β FACTORS

Recombinant hybrid TGF- β 1/ β 2 was produced as
described in Section 6, et seq, supra. TGF- β 1 and
35 TGF- β 2 were prepared, respectively, as described in

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Assoian et al., 1983, J. Biol. Chem. 258:7155-60, and
in Cheifetz et al., 1987, Cell 48:409-15, and were
obtained from Drs. A. Roberts and M. Sporn at the
Laboratory of Chemoprevention, National Cancer
5 Institute, Bethesda, MD.

7.1.3 MATRIX COMPONENTS

Tissue culture dishes and flasks were coated with
purified bovine type I collagen (Madri and Furthmayr,
10 1980, Hum. Path. 11:353-66) at a concentration of 12.5
 $\mu\text{g/ml}$.

7.1.4 PROLIFERATION INHIBITION ASSAY

Collagen type I-coated dishes were washed in PBS
15 before the addition of cell suspensions (1.4×10^4
cells/dish). After 6 h, the "initial" number of cells
on the substrate was determined. At this time point,
fresh medium with or without TGF- β s was added to the
cultures. Medium and TGF- β s were replaced once again
20 on day 3. Cell numbers were determined by lifting the
cells off the culture dishes with trypsin/EDTA and
counting quadruplicate samples using a Coulter counter
(Coulter Electronics, Inc., Hialeah, FL). The mean
number of cells per dish for each TGF- β addition was
25 then calculated.

7.1.5 CELL MIGRATION ASSAYS

Stimulus for cell migration was accomplished by
releasing confluent cultures from contact inhibition
30 using a stainless steel "fence" device as previously
described (Pratt et al., 1984, Am. J. Path. 117:349-
54). Once confluency had been achieved in the center
wells of the fences (~6 h), migration was induced by
removing the fences, and cells were thereafter
35 observed migrating outward in a radial fashion.

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Cultures were fed once again on day 3 with control or TGF- β -supplemented media. After 6 days, the cultures were washed with PBS, fixed with 10% neutral buffered formalin and stained with Harris hematoxylin. Net
5 increase in surface area covered was assessed using an overhead projector, and areas were calculated utilizing a computerized graphics tablet and "MacMeasure" software (Yale Shareware, Yale
Univeristy, New Haven, CT).

10

7.2 RESULTS

The majority of published reports indicate that the TGF- β 1 and TGF- β 2 isoforms are equipotent in vitro. However, recent reports indicate that these
15 isoforms are not functionally interchangeable, since they elicit dissimilar effects and exhibit different spatial and temporal localizations (See, for example, Ohto et al., 1987, Nature 329:539-41; Rosa et al., 1988, Science 239:783-85; and Danielpour et al., 1989,
20 J. Cell Physiol. 138:79-86). TGF- β 1 is reportedly more effective than TGF- β 2 in inhibiting DNA synthesis in endothelial cells (Jennings, et al., 1988, J. Cell. Physiol. 137:167-72).

A recent report establishes that TGF- β 1 and TGF-
25 β 2 isoforms elicit differential responses in proliferation assays using three vascular cell types (Merwin et al., 1991, Am J. Path. 138(1):37-51). Therefore, investigating the effects of TGF-5 β on endothelial cells seemed ideal for determining whether
30 the TGF- β 1/TGF- β 2 hybrid (TGF-5 β) would evoke responses similar to TGF- β 1 or to TGF- β 2. The results indicated that TGF-5 β mimicked TGF- β 1 in all three proliferation assays examined, while TGF- β 2 displayed unique properties.

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Both TGF- β 1 and TGF- β 2 inhibited BAEC proliferation by more than 70% (\pm 1.5%) at the optimum concentration of 0.5 ng/ml (FIG. 4A). In contrast, TGF- β 2 was only able to evoke inhibition of about 20% (\pm 2.5%). When using BASMCs, all three growth factors exhibited similar inhibitory activities (FIG. 4B). Microvascular endothelial cells were growth inhibited as much as 70% (\pm 4.0%) by both TGF- β 1 and TGF- β 2 at concentrations of 0.5 ng/ml (FIG. 4C). At equivalent concentration, however, TGF- β 2 was only able to mount a 37% (\pm 8%) inhibition; however, with a 10-fold increase in concentration, TGF- β 2 was able to evoke a similar response.

Similarly, hybrid TGF- β 3 exerted a cell migration response equivalent to that induced by TGF- β 1. The results presented in FIG. 5 show that BAECs are inhibited by both TGF- β 3 and TGF- β 1, while BASMCs are growth-stimulated by both factors. In contrast, TGF- β 2 had no effect on the migration of either cell type.

The above results may help to explain vascular response to injury. Upon denudation (*i.e.*, following angioplasty) of the endothelial barrier, platelets adhere and aggregate to the newly exposed subendothelial extracellular matrix and release a variety of molecules. In this regard, one of the major factors released by platelets is TGF- β 1. With a marked increase in the local concentration of TGF- β 1, endothelial cells may be inhibited from migrating to affect a rapid re-endothelialization of the injury while at the same time, smooth muscle cell migration toward an increased factor gradient may be stimulated, thereby establishing an ideal opportunity for perpetuating the expansion of the medial compartment of the vessel wall and continued occlusion of the vascular lumen.

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While angiogenesis is an important and necessary response during embryogenesis and tissue repair, it is an undesirable phenomenon in the context of solid tumor growth. Therefore, understanding the

5 enhancement of neovascularization is of paramount concern. To investigate the TGF- β -induced angiogenic response in microvascular endothelial cells, RFCs were grown in three-dimensional collagen cultures for 4 days, either in the presence or absence of TGF- β

10 isoforms. All three isoforms were able to stimulate RFCs to mount a neovascularization response. This endothelial "activation" was previously defined by extensive EM analysis (Merwin et al., 1990, J. Cell. Physiol. 142:117-29) which revealed the formation of

15 branching tubular structures, cell-cell junctional complexes, metabolic stimulation (increased numbers of ribosomes and engorged ER), cellular polarity, deposition and organization of extracellular matrix proteins and lumen formation.

20 FIG. 6A shows control cultures with no exogenous growth factor. Cells remain independent of one another and lack tubular structures, while treatment with TGF- β 1 (FIG. 6B) reveals complex, tube-like formations. TGF- β 2 (FIG. 6C) was able to initiate a

25 modest angiogenic response. However, as with RFC proliferation studies, an increase in TGF- β 2 concentration from 0.5 to 5.0 ng/ml was necessary for the establishment of complex tubular structures. TGF- β 5 mimicked TGF-1, with tube formation initiated with

30 as little as 0.05 ng/ml (FIG. 6D), and optimal neovascularization achieved at a concentration of 0.5 ng/ml (FIG. 6E). A slight inhibition of the angiogenic response was noted at the highest concentration used, 5.0 ng/ml (FIG. 6F).

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In summary, TGF- β 5 mimics TGF- β 1 in inhibiting proliferation of low density monolayer micro and large vessel endothelial cell cultures by about 70% and by about 45% on BASMCs. In contrast, an equivalent concentration of TGF- β 2 is ineffective with BAECs and equivalent on BASMCs. Moreover, a 10-fold increase in TGF- β 2 concentration is required to elicit a similar response with RFCs. When using a culture which begins with a tight, confluent monolayer, response induced by the isoforms differ. In particular, both TGF- β 1 and TGF- β 2 induce equivalent levels of BASMC migration enhancement and BAEC migration inhibition, while TGF- β 2 does not influence the migration of either cell type. Finally, in microvascular endothelial cells which respond to injury in vivo by neovascularization, all three TGF- β s were able to stimulate angiogenesis in vitro. However, a 10-fold increase in TGF- β 2 concentration was necessary to match the level of response induced by TGF- β 1 and TGF- β 5.

8. DEPOSIT OF MICROORGANISMS

The following transfectant has been deposited with the American Type Culture Collection, Rockville, MD, and has been assigned the listed accession number.

| <u>Transfectant</u> | <u>Plasmid</u> | <u>Accession No.</u> |
|---------------------------|------------------|----------------------|
| CHO-5 β 41,2.5 CL 5 | p5 β /dhfr | CRL 9959 |

The present invention is not to be limited in scope by the cell line deposited or the embodiments disclosed herein which are intended as single illustrations of one aspect of the invention and any which are functionally equivalent are within the scope of the invention. Indeed, various modifications of the invention, in addition to those shown and

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described herein, will become apparent to those skilled in the art from the foregoing description. Such modifications are intended to fall within the scope of the appended claims.

5 It is also to be understood that all base pair and amino acid residue numbers and sizes given for nucleotides and peptides are approximate and used for the purposes of description.

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WHAT IS CLAIMED IS:

1. A pharmaceutical composition comprising
chimeric transforming growth factor $\beta 1/\beta 2$ for use in
5 inhibiting the proliferation of vascular endothelial
cells.
2. A pharmaceutical composition comprising
chimeric transforming growth factor $\beta 1/\beta 2$ for use in
10 inducing smooth muscle cell migration.
3. A pharmaceutical composition comprising an
anti-chimeric transforming growth factor $\beta 1/\beta 2$
antibody for use in inhibiting microvascular cell
15 neovascularization.

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| | | |
|-----------------|--|--|
| SIMIAN HUMAN | -261 | AGGGGATCTGTGGCAGGTCGGAGA--AAGA -234 CTCC..C...CCA...A..CCCT.TTC.... |
| SIMIAN HUMAN | TC---CGTCTCCTGGTACCAGATCTCGCCCATCTAGGTTATTCCGTGGGATACTGAGACACCCCGGTCCAAGCCTCC -158 C.ACC.AC..T.....G..... | |
| SIMIAN HUMAN | CCTCCACCACCTGGCCCTTCTCCCTGAGGA-CCTCAACTTTCCTCGAGGCCCTCCTACCTTTCCGGGGACCCCA -80G.....G.....A..... | |
| SIMIAN HUMAN | <u>GCCCCCTGCAGGGCGGGCCTCCCCACCAAACTAGCCCTGTTCGGCCTCTCGGCAGTGCCGGGGCGCCCTCCCCC -1</u>C..C..... | |
| SIMIAN HUMAN | Met Pro Pro Ser Gly Leu Arg Leu Leu Pro Leu Leu Trp Leu Leu Val 20 60 ATG CCG CCC TCC GGG CTG CGG CTG CTG CCG CTG CTA CCA CTG CTG TGG CTA CTG GTG Leu Thr Pro Ser Arg Pro Ala Ala Gly Leu Ser Thr Cys Lys Thr Ile Asp Met Glu Leu 40 120 CTG ACG CCT AGC CGG CCG GCA GGA CTA TCC ACC TGC AAG ACT ATC GAC ATG GAG CTGG.. .C. | |
| SIMIAN HUMAN | Val Lys Arg Lys Arg Ile Glu Thr Ile Arg Gly Gln Ile Leu Ser Lys Leu Arg Leu Ala 60 180 GTG AAG CCG AAG CAG CGC ATC GAG ACC ATC CGC GGC CAG ATC CTG TCC AAG CTG CGG CTC GCCG... Ser Pro Pro Ser Gln Gly Glu Val Pro Pro Gly Pro Leu Pro Glu Ala Val Leu Ala Leu 80 240 AGC CCC CCG AGC CAG GGG GAG GTG CCG CCC GGC CCG CTG CCC GAG GCC CTC GCC CTG | |

FIG.1A

| | | | | | | | | | | | | | | | | | | | | | | |
|-----------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| SIMIAN HUMAN | Tyr | Asn | Ser | Thr | Arg | Arg | Val | Ala | Gly | Glu | Ser | Ala | Glu | Pro | Glu | Pro | Glu | Pro | Glu | 100 | | |
| | TAC | AAC | AGC | ACC | CGC | GAC | GTG | GCC | GGG | GAG | AGT | GCG | GAG | CCG | GAG | CCC | GAA | CCG | GAG | 300 | | |
| | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ..A | ... | ... | ... | ... | ..G | ..T | ... | | | |
| SIMIAN HUMAN | Ala | Asp | Tyr | Tyr | Ala | Lys | Glu | Val | Thr | Arg | Val | Leu | Met | Val | Glu | Thr | His | Asn | Glu | 120 | | |
| | GCC | GAC | TAC | TAC | GCC | AAG | GAG | GTC | ACC | CGC | GTG | CTA | ATG | GTG | GAA | ACC | CAC | AAC | GAA | 360 | | |
| | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | | |
| SIMIAN HUMAN | Tyr | Asp | Lys | Phe | Lys | Gln | Ser | Thr | His | Ser | Ile | Tyr | Met | Phe | Phe | Asn | Thr | Ser | Glu | 140 | | |
| | TAT | GAC | AAG | TTC | AAG | CAG | AGC | ACA | CAC | AGC | ATA | TAT | ATG | TTC | TTC | AAC | ACA | TCA | GAG | 420 | | |
| | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | | |
| SIMIAN HUMAN | Arg | Glu | Ala | Val | Pro | Glu | Pro | Val | Leu | Leu | Ser | Arg | Ala | Glu | Leu | Arg | Leu | Leu | Arg | 477 | | |
| | CGA | GAA | GCA | GTA | CCT | GAA | CCT | GTG | TTG | CTC | TCC | CGG | GCA | GAG | CTG | CGT | CTG | CTG | --- | Arg | | |
| | ... | ... | ..G | ... | ... | ... | ..C | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | --- | AGG | | |
| SIMIAN HUMAN | 160 | Leu | Lys | Leu | Lys | Val | Glu | Gln | His | Val | Glu | Tyr | Gln | Lys | Tyr | Ser | Asn | Asn | Ser | Trp | 537 | |
| | CTC | AAG | TTA | AAA | GTG | GAG | CAG | CAT | GTG | GAG | CTG | TAC | CAG | AAA | TAC | AGC | AAC | AAT | TCC | TGG | | |
| | ... | ... | ... | ... | ... | ... | ... | ..C | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | | |
| SIMIAN HUMAN | 180 | Arg | Tyr | Leu | Ser | Asn | Arg | Leu | Leu | Ala | Pro | Ser | Asn | Ser | Pro | Glu | Trp | Leu | Ser | Phe | Asp | 597 |
| | CGA | TAC | CTC | AGC | AAC | CGG | CTG | CTG | GCG | CCC | AGC | AAC | TCG | CCG | GAG | TGG | TTG | TCT | TTT | GAT | | |
| | ... | ... | ... | ... | ... | ... | ... | ... | ..A | ... | ... | G.. | ... | ..A | ... | ... | ..A | ... | ... | ... | ... | |

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FIG.1B

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[illegible]

FIG. 1C

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FIG. 1D

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FIG. 2A

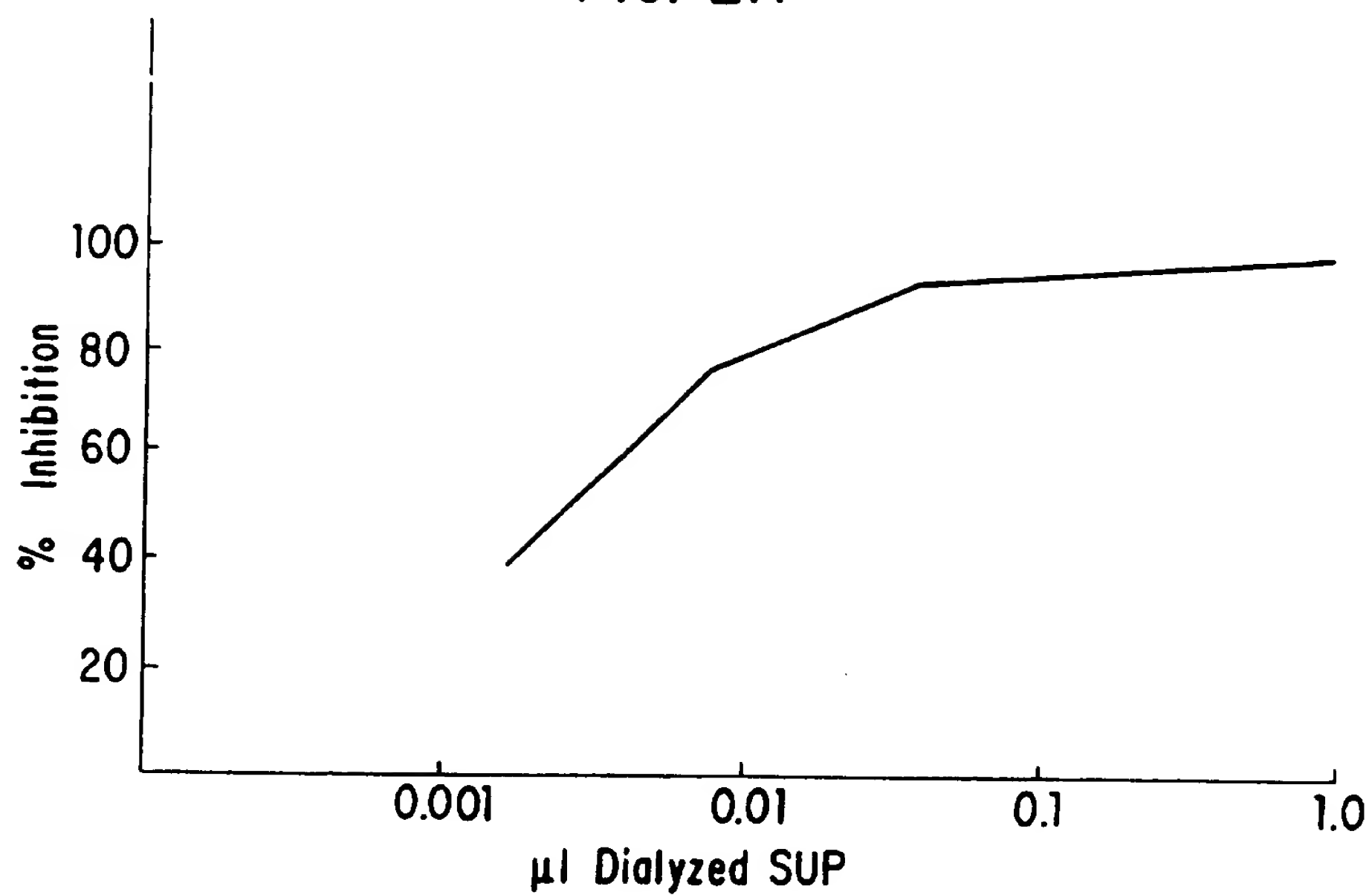
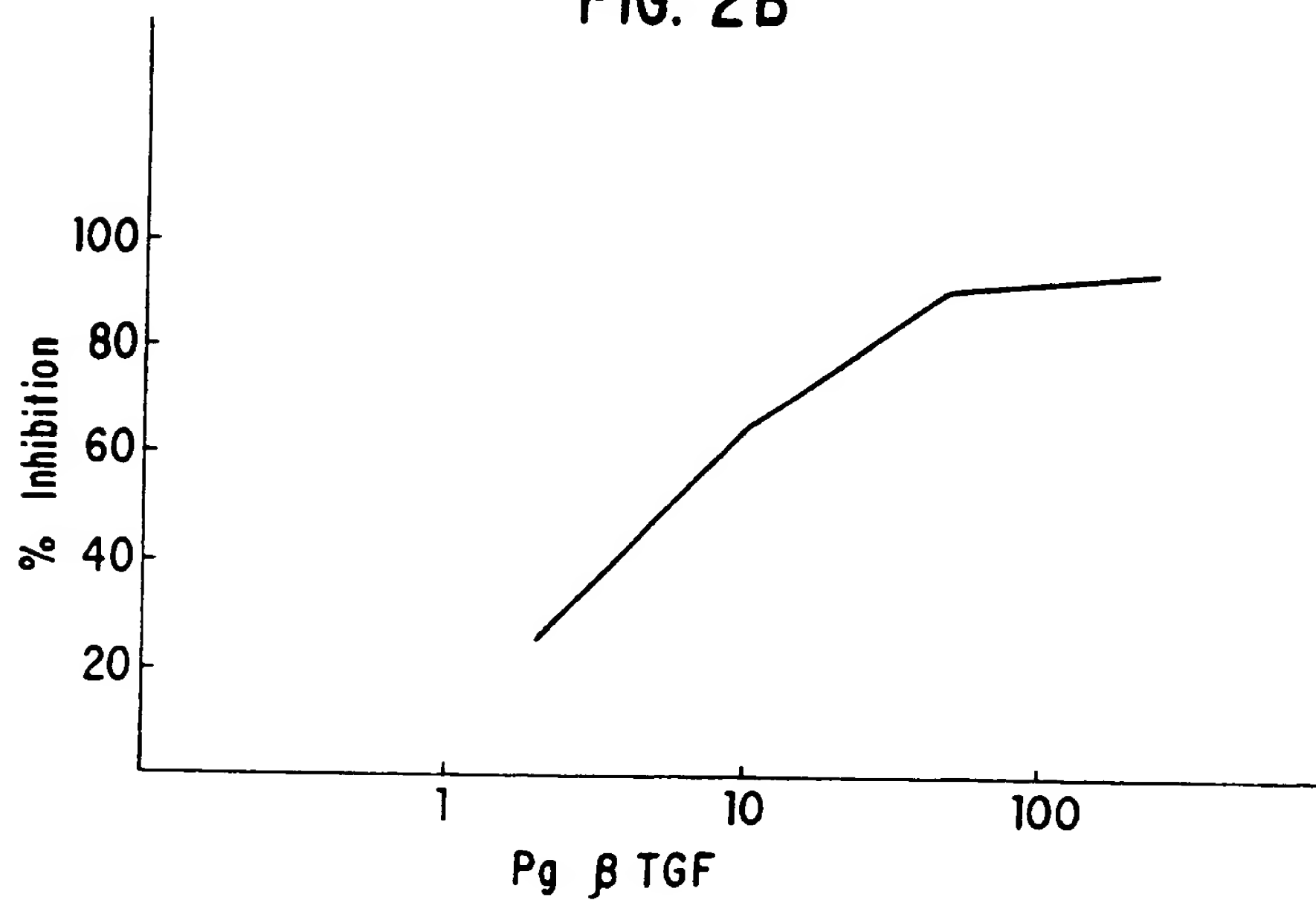


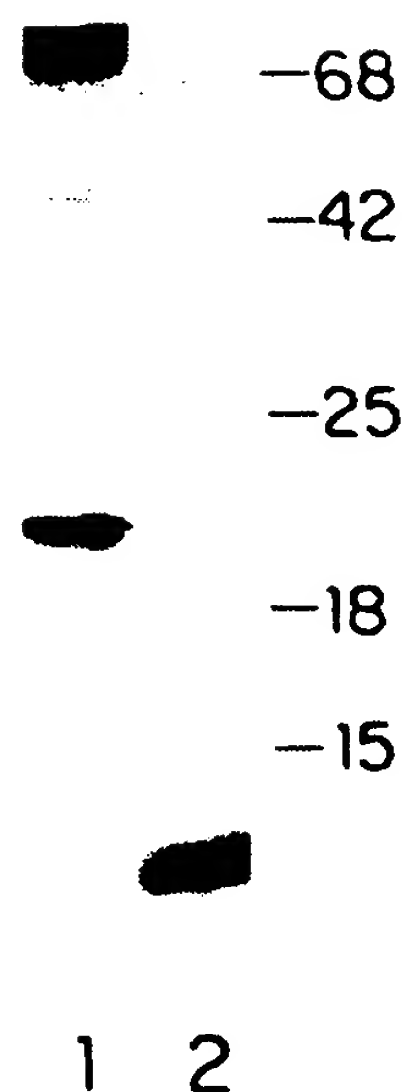
FIG. 2B



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FIG. 3



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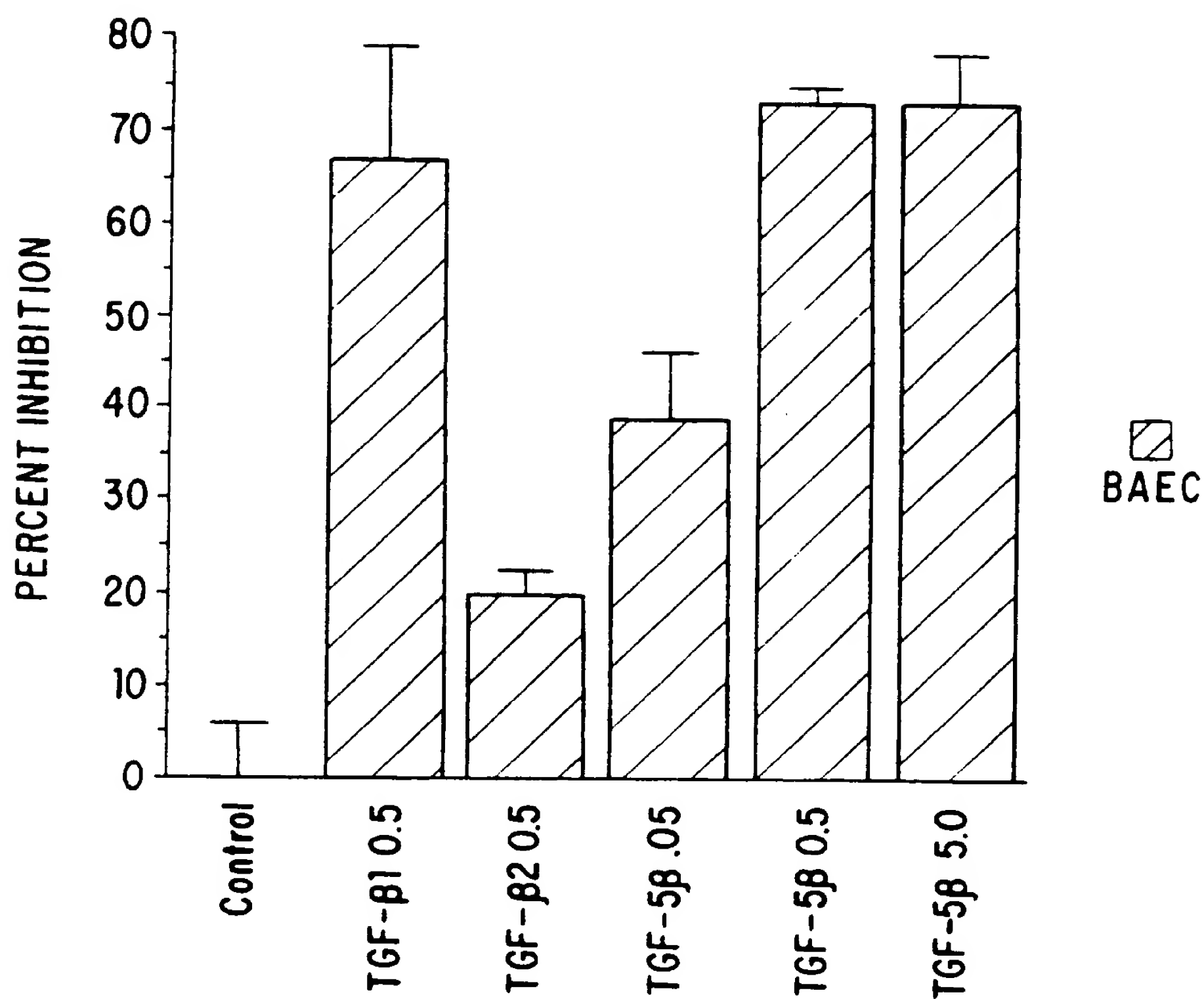


FIG. 4A

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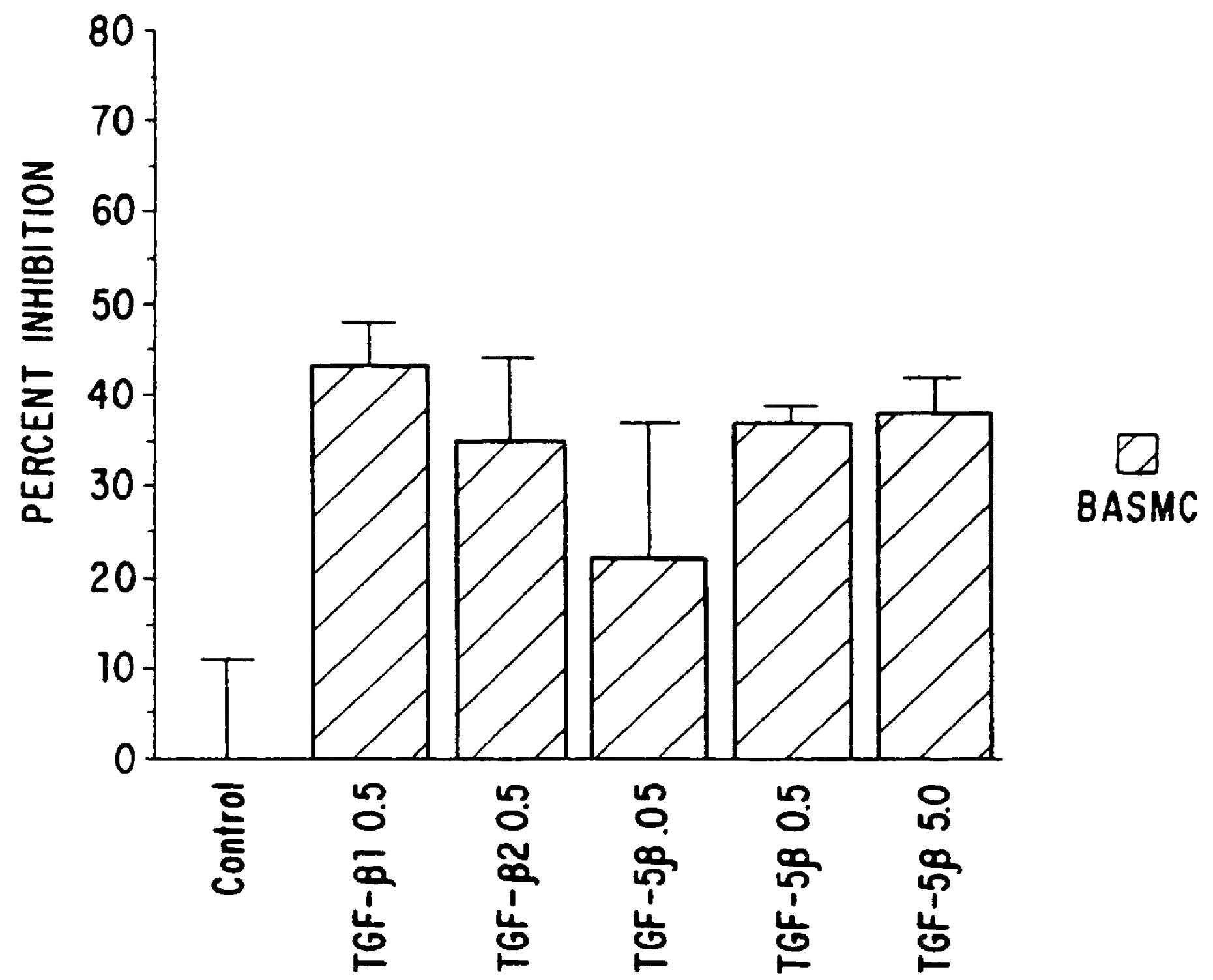


FIG. 4B

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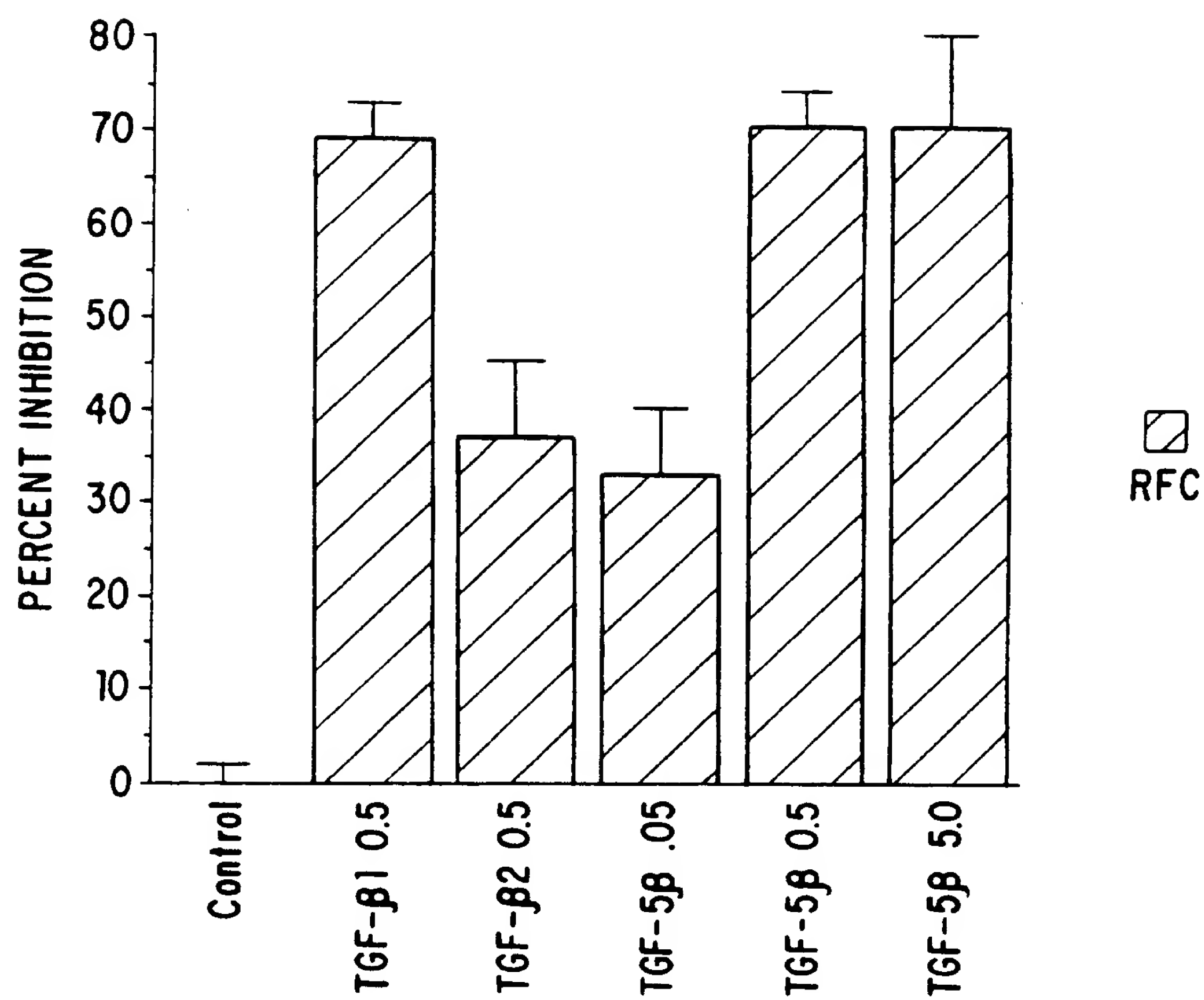


FIG. 4C

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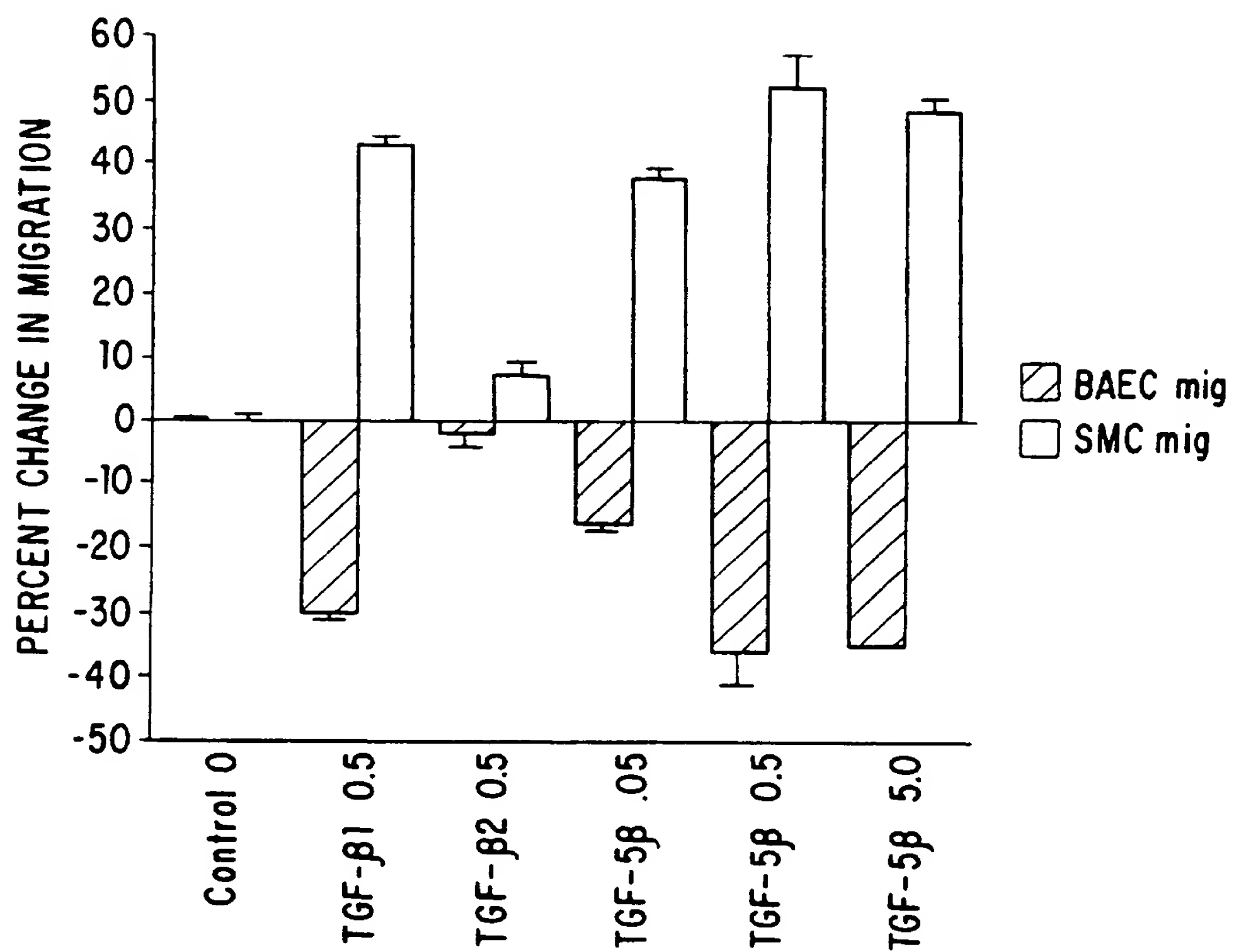


FIG. 5

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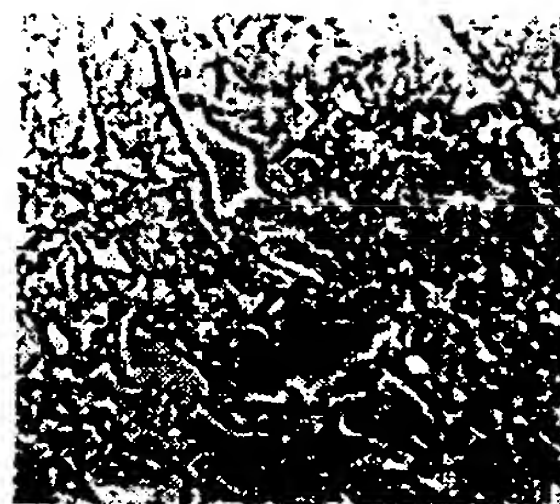


FIG. 6A

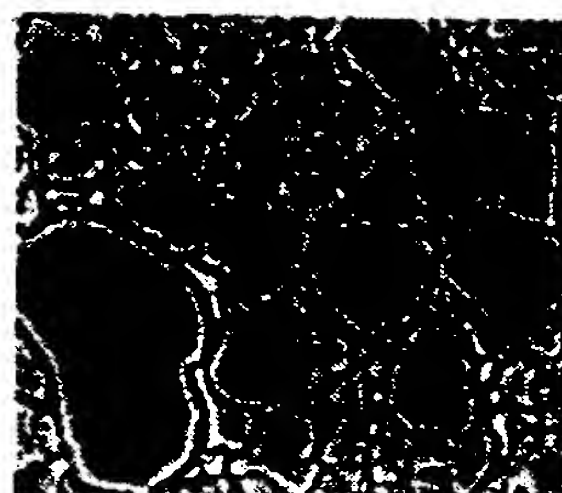


FIG. 6B

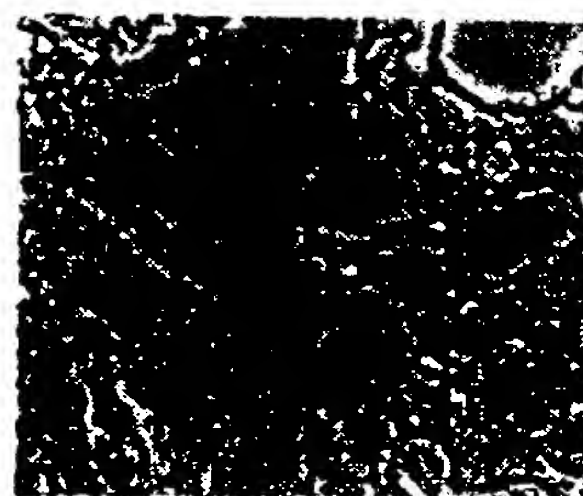


FIG 6C

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FIG. 6D

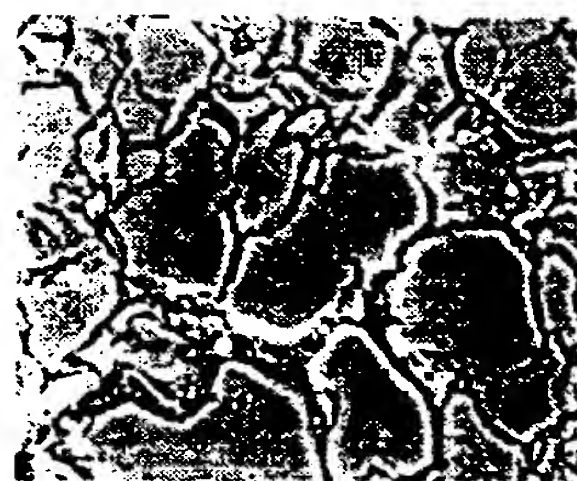


FIG. 6E

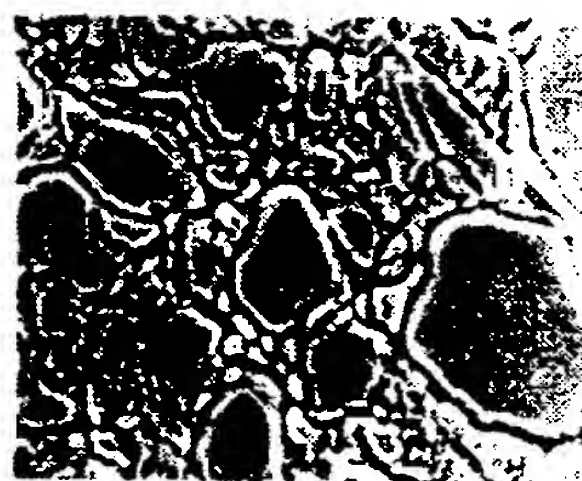


FIG 6F

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US92/01993

| I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ³ According to International Patent Classification (IPC) or to both National Classification and IPC IPC (5): A61K 37/24; C07K 13/00 US CL : 514/12; 530/399 | | | | | | | | | | | | | | |
|--|--|-------------------------------------|--|---|--|---|---|-----|---|--|-----|-----|--|-----|
| II. FIELDS SEARCHED <div style="text-align: center; border: 1px solid black; padding: 2px; margin: 5px 0;">Minimum Documentation Searched⁴</div> <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 30%; border: 1px solid black; padding: 2px;">Classification System</th> <th style="border: 1px solid black; padding: 2px;">Classification Symbols</th> </tr> <tr> <td style="border: 1px solid black; text-align: center; padding: 5px;">U.S.</td> <td style="border: 1px solid black; padding: 5px;">514/12; 530/399</td> </tr> </table> <div style="text-align: center; border: 1px solid black; padding: 2px; margin: 5px 0;">Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched⁵</div> DIALOG, MEDLINE, APS, PROTEIN SEQUENCE DATABASES (PIR, SWISS-PROT) | | | Classification System | Classification Symbols | U.S. | 514/12; 530/399 | | | | | | | | |
| Classification System | Classification Symbols | | | | | | | | | | | | | |
| U.S. | 514/12; 530/399 | | | | | | | | | | | | | |
| III. DOCUMENTS CONSIDERED TO BE RELEVANT¹⁴ <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 10%; border: 1px solid black; padding: 2px;">Category⁶</th> <th style="border: 1px solid black; padding: 2px;">Citation of Document¹⁶ with indication, where appropriate, of the relevant passages¹⁷</th> <th style="width: 10%; border: 1px solid black; padding: 2px;">Relevant to Claim No.¹⁸</th> </tr> <tr> <td style="border: 1px solid black; text-align: center; vertical-align: top; padding: 5px;">A</td> <td style="border: 1px solid black; padding: 5px;">US, A, 4,742,003 (Derynck, et al.) 03 MAY 1988.</td> <td style="border: 1px solid black; text-align: center; vertical-align: top; padding: 5px;">1-3</td> </tr> <tr> <td style="border: 1px solid black; text-align: center; vertical-align: top; padding: 5px;">A</td> <td style="border: 1px solid black; padding: 5px;">J. BIOL. CHEM., Volume 265, issued 05 November 1990, R. Lafyatis, "Structural and Functional Characterization of the Transforming Growth Factor beta 3 Promotor. A cAMP-Responsive Element Regulates Basal and Induced Transcription," pages 19128-36.</td> <td style="border: 1px solid black; text-align: center; vertical-align: top; padding: 5px;">1-3</td> </tr> <tr> <td style="border: 1px solid black; text-align: center; vertical-align: top; padding: 5px;">A,E</td> <td style="border: 1px solid black; padding: 5px;">CLINICAL RESEARCH, volume 40, APRIL 1992, A.B. Kulkarni, "Mouse Chimeras With A Disrupted Transforming Growth-Beta-1 Gene Obtained By Gene Targeting", page 295, N2.</td> <td style="border: 1px solid black; text-align: center; vertical-align: top; padding: 5px;">1-3</td> </tr> </table> | | | Category ⁶ | Citation of Document ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷ | Relevant to Claim No. ¹⁸ | A | US, A, 4,742,003 (Derynck, et al.) 03 MAY 1988. | 1-3 | A | J. BIOL. CHEM., Volume 265, issued 05 November 1990, R. Lafyatis, "Structural and Functional Characterization of the Transforming Growth Factor beta 3 Promotor. A cAMP-Responsive Element Regulates Basal and Induced Transcription," pages 19128-36. | 1-3 | A,E | CLINICAL RESEARCH, volume 40, APRIL 1992, A.B. Kulkarni, "Mouse Chimeras With A Disrupted Transforming Growth-Beta-1 Gene Obtained By Gene Targeting", page 295, N2. | 1-3 |
| Category ⁶ | Citation of Document ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷ | Relevant to Claim No. ¹⁸ | | | | | | | | | | | | |
| A | US, A, 4,742,003 (Derynck, et al.) 03 MAY 1988. | 1-3 | | | | | | | | | | | | |
| A | J. BIOL. CHEM., Volume 265, issued 05 November 1990, R. Lafyatis, "Structural and Functional Characterization of the Transforming Growth Factor beta 3 Promotor. A cAMP-Responsive Element Regulates Basal and Induced Transcription," pages 19128-36. | 1-3 | | | | | | | | | | | | |
| A,E | CLINICAL RESEARCH, volume 40, APRIL 1992, A.B. Kulkarni, "Mouse Chimeras With A Disrupted Transforming Growth-Beta-1 Gene Obtained By Gene Targeting", page 295, N2. | 1-3 | | | | | | | | | | | | |
| <div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>¹⁵ Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&" document member of the same patent family</p> </div> </div> | | | | | | | | | | | | | | |
| IV. CERTIFICATION <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; border: 1px solid black; padding: 5px;"> Date of the Actual Completion of the International Search² <div style="text-align: center; font-weight: bold;">22 June 1992</div> </td> <td style="width: 50%; border: 1px solid black; padding: 5px;"> Date of Mailing of this International Search Report² <div style="text-align: center; font-weight: bold;">30 JUN 1992</div> </td> </tr> <tr> <td style="border: 1px solid black; padding: 5px;"> International Searching Authority¹ <div style="text-align: center; font-weight: bold;">ISA/US</div> </td> <td style="border: 1px solid black; padding: 5px;"> Signature of Authorized Officer²⁰ <div style="text-align: center;"> SHELLY J. GUEST </div> </td> </tr> </table> | | | Date of the Actual Completion of the International Search ² <div style="text-align: center; font-weight: bold;">22 June 1992</div> | Date of Mailing of this International Search Report ² <div style="text-align: center; font-weight: bold;">30 JUN 1992</div> | International Searching Authority ¹ <div style="text-align: center; font-weight: bold;">ISA/US</div> | Signature of Authorized Officer ²⁰ <div style="text-align: center;"> SHELLY J. GUEST </div> | | | | | | | | |
| Date of the Actual Completion of the International Search ² <div style="text-align: center; font-weight: bold;">22 June 1992</div> | Date of Mailing of this International Search Report ² <div style="text-align: center; font-weight: bold;">30 JUN 1992</div> | | | | | | | | | | | | | |
| International Searching Authority ¹ <div style="text-align: center; font-weight: bold;">ISA/US</div> | Signature of Authorized Officer ²⁰ <div style="text-align: center;"> SHELLY J. GUEST </div> | | | | | | | | | | | | | |

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